

REMARKS

I. Status Summary

Claims 23-30 and 32-46 are pending in the present application and have been examined by the United States Patent and Trademark Office (hereinafter "the Patent Office").

Claims 26, 29, 35, 41, and 42 have been objected to as being dependent from a rejected base claim.

Claims 23, 27, 28, 30, 32-34, 37-40, and 43-46 have been rejected under 35 U.S.C. § 102(e) upon the contention that they are anticipated by U.S. Patent Application Publication No. 2002/0168351 of Ohno (hereinafter "Ohno").

Claims 23, 25, 27, 28, 30, 32-34, 36-40, and 43-46 have been rejected under 35 U.S.C. § 103(a) upon the contention that the claims are unpatentable over Ohno in view of PCT International Patent Application Publication No. WO 97/41210 of Nair et al. (hereinafter "Nair"). Claims 23-25, 27, 28, 30, 32-34, and 36-46 have also been rejected under this section upon the contention that they are unpatentable over Ohno and Nair and further in view of U.S. Patent No. 6,077,519 to Storkus et al. (hereinafter "Storkus"). Claims 23, 27, 28, 30, 32-34, 37-40, and 43-46 have also been rejected under this section upon the contention that they are unpatentable over Ohno.

Claims 23, 27, and 33 have been amended. Support for the amendments can be found throughout the specification as filed, including particularly at page 7, lines 14-17. Thus, no new matter has been added by the amendments to the claims.

Reconsideration of the application as amended and in view of the remarks set forth herein is respectfully requested.

II. Discussion Regarding the Presently Disclosed Subject Matter

The presently disclosed subject matter relates *inter alia* to compositions comprising semi-allogeneic, HLA-haploididentical antigen-presenting cells (APCs), and methods for using the same. The APCs are donor cells that match a single HLA-haplotype of a recipient, but that do not match the recipient's second HLA-haplotype (hence, semi-allogeneic). Applicants respectfully submit having the non-HLA-matched haplotype in the APCs so that strong allogenic stimulation by mismatched MHC

molecules present on the APCs can support induction of strong immune responses, including activation of innate immune cells is desirable.

The Patent Office acknowledges that matching for HLA-haplotypes in autologous cells is important. However, applicants respectfully submit that the Patent Office has not considered or accounted for the fact that there is no difference in HLA expression in autologous transfers. For example, the Patent Office recognizes that autologous donor cells have two identical HLA-haplotypes with the recipient patient. This means that all of the innate immune cells of the patient that can be negatively regulated by self-HLA molecules (*i.e.*, natural killer cells and natural killer-like T cells) would be expected to encounter a "self" HLA molecule on the surface of every transferred autologous cells. This would result in the transferred autologous cells receiving negative regulatory (*i.e.*, inhibitory) signals. Even if new allogeneic HLA molecules are introduced into these autologous cells (*e.g.*, via fusion with allogenic tumor cells, introduction of RNA, DNA, proteins, etc.), the negative regulation signal would still be delivered since the autologous cells express the full complement of HLA molecules encoded by the two autologous HLA-haplotypes (*i.e.*, the paternal and maternal HLA haplotypes) of the patient. This fact is conceded by the Patent Office on page 6, last line in paragraph 1, of the Final Official Action. The allogenic molecules are thus merely an "add-on" to the HLA molecules encoded by the two autologous HLA-haplotypes; they will not displace the autologous HLA molecules encoded by the two autologous HLA-haplotypes.

Thus, in an autologous transfer, every antigen-presenting cell expresses the full complement of HLA molecules of the patient (encoded by the paternal and maternal HLA alleles) and as such would receive negative regulatory signals that inhibit their activities once transferred into the patient.

On the contrary, with HLA-haploidentical cells, only one HLA-haplotype is identical with the patient. The other HLA-haplotype is different. This means that only about half of the repertoire of innate immune effector cells (NK cells and NK-like T cells) would encounter an HLA molecule of the patient on the antigen-presenting cells that can negatively regulate them (*i.e.*, HLA molecules encoded by the HLA-haplotype that is shared between the HLA-haploidentical antigen-presenting cells and the patient). The remaining NK cells and NK-like cells of the patient would not encounter self HLA

molecules on the transferred antigen-presenting cells, and thus their activities would not be negatively regulated.

Because it is genetically determined by the HLA constellation, one HLA-haplotype is shared with the patient and the other HLA-haplotype is not shared by the patient, which is what characterizes semi-allogeneic, HLA-haploidentical cells. Thus, the presently disclosed and claimed antigen-presenting cells have the capacity to activate MHC-restricted (*i.e.*, HLA-restricted) T cell responses through presentation of peptides by shared HLA molecules due to the presence of one shared HLA-haplotype as well as the capacity to allow some innate NK cells and NK-like cells to develop immune responses since they do not encounter the full complement of autologous HLA molecules of the patient that are needed to negatively regulate them. Further, negative inhibitory receptors are not shared by all NK cells. Rather, individual NK cells express different NK receptors. Therefore, to negatively regulate the entire population of NK cells one must have the entire complement of self-HLA molecules. This condition is not met when semi-allogeneic HLA-haploidentical cells are used since only half of the HLA molecules of the patient are present, thereby allowing NK activity to take place.

### III. Response to the Anticipation Rejection

Claims 23, 27, 28, 30, 32-34, 37-40, and 43-46 have been rejected under 35 U.S.C. § 102(e) upon the contention that they are anticipated by U.S. Patent Application Publication No. 2002/0168351 of Ohno (hereinafter "Ohno"). This rejection has been maintained from the previous Non-Final Official Action.

After careful consideration of the rejection and the Patent Office's basis therefor, applicants respectfully traverse the rejection and submit the following remarks.

According to the Patent Office, Ohno teaches using APCs that are autologous. The Patent Office further asserts that autologous cells from a patient would have two identical haplotypes to the patient. Thus, it appears that the Patent Office is asserting that autologous APCs are haploidentical APCs.

Applicants respectfully disagree. Applicants respectfully submit that the definition of "haplotype" that the Patent Office cited on page 6 of the Final Official Action from The Dictionary of Immunology (Third Edition, 1985) does not support the instant rejection.

First, the definition relied on by the Patent Office is not of a term recited in the instant claims, and thus is of little value in interpreting the claims.

Second, the Patent Office apparently concludes that "haploidentical" is equivalent to "having at least one identical haplotype", which leads the Patent Office to assert that cells that have two identical haplotypes (e.g., autologous cells) are also "haploidentical". Applicants respectfully submit that this conclusion is unsupported by any authority and is inconsistent with both how the term "haploidentical" has been employed in the art as well as how it would be understood by one of ordinary skill in the art after consideration of the instant specification as a whole.

To elaborate, applicants respectfully submit that the term "haploidentical" refers to donors and recipients that are identical with respect to each other in exactly one haplotype. Applicants further respectfully submit that donors and recipients that are identical with respect to each other in two haplotypes are referred to as "autologous" or "syngeneic". The Patent Office has provided no support for its contention that autologous cells such as those set forth in Ohno would be considered "haploidentical" cells, and thus the Patent Office's attempts to equate "haploidentical" and "identical haplotypes" is believed to be in error.

Furthermore, applicants respectfully submit that they are employing the term "haploidentical" in a manner that is consistent with its use in the art. For example, several publications draw a distinction between "autologous" and "haploidentical". Examples of such a distinction are provided herewith as **Exhibits A-C**. With respect to the **Exhibits**, the Patent Office's attention is directed to page 861 of **Exhibit A** (Table I and the discussion of *Group A* and *Group B* in the right hand column); the Summary of **Exhibit B** ("autologous marrow mixed with HLA-haploidentical allogeneic marrow"; emphasis added); and col. 6, lines 26-28 and col. 14, lines 12-15 of **Exhibit C** (contrasting the terms "syngeneic" and "haploidentical"). In each case, the reference shows that the terms "autologous" (or "syngeneic") and "haploidentical" refer to different cells. Thus, applicants respectfully submit that at the time of filing, those of skill in the art employed the term "haploidentical" to refer to a cell that is distinct from an autologous cell.

Additionally, applicants respectfully submit that they are employing the term "haploidentical" in the instant specification in a manner that is consistent with its ordinary and customary meaning in the art. According to the Court of Appeals for the Federal Circuit ("Federal Circuit"), unless a claim term is specifically defined otherwise in the specification, it must be given its ordinary and customary meaning. The Federal Circuit further held that the ordinary and customary meaning is "the meaning that the term would have to a person of ordinary skill in the art in question at the time of the invention, i.e., as of the effective filing date of the patent application." (*citing Phillips v. AWH Corp.*, 415 F.3d 1303, 1313, 75 USPQ2d 1321, 1326 (Fed. Cir. 2005) (*en banc*). *Sunrace Roots Enter. Co. v. SRAM Corp.*, 336 F.3d 1298, 1302, 67 USPQ2d 1438, 1441 (Fed. Cir. 2003); *Brookhill-Wilk 1, LLC v. Intuitive Surgical, Inc.*, 334 F.3d 1294, 1298 67 USPQ2d 1132, 1136 (Fed. Cir. 2003)). See also M.P.E.P. § 2111.11.

Applicants respectfully submit that the "ordinary and customary meaning" of "haploidentical" as reflected in the specification and in the art (exemplified by **Exhibits A-C**) is a donor cell that is allogeneic to the recipient, but that has class I and class II HLA molecules in common with the recipient. This makes the cells semi-allogeneic. This specific characteristic is reflected in the instant specification at page 6, line 32, through page 7, line 24.

Furthermore, applicants respectfully submit that the instant specification specifically distinguishes between "haploidentical" and "autologous". Particularly, page 18 of the instant specification includes a section entitled "Comparison of autologous versus HLA-haploidentical dendritic cells as antigen-presenting cells". The bridging paragraph between pages 18 and 19 states the following:

The experiments analyzing the role of HLA-haploidentical dendritic cells will be done by comparing autologous dendritic cells prepared from a normal control donor (donor #1) and HLA-haploidentical dendritic cells prepared from a selected HLA-typed family member (donor #2) (i.e. a parent or sibling is selected to have one HLA haplotype in common with donor 1 and the second HLA-haplotype is mismatched; see Fig. 2).

Specification at page 18, line 29, through page 19, line 3 (emphasis added).

Therefore, applicants respectfully submit that when one of ordinary skill in the art considers the instant application as a whole, it would be clear that the term

"haploidentical" does not include "autologous", and in fact specifically excludes "autologous". To clarify this point, claims 23, 27, and 33 have been amended to recite that the APCs are semi-allogeneic HLA-haploidentical APCs. Support for the amendments can be found throughout the specification as filed, including particularly at page 7, lines 14-17. Thus, no new matter has been added by the amendments to the claims.

Summarily, applicants respectfully submit that the Patent Office has adopted an improperly broad meaning for the term "haploidentical" that is inconsistent with how that term is employed in the art and is further inconsistent with how the term is employed in the instant specification. Applicants further respectfully submit that when the term is given its art-recognized meaning, which is consistent with the use of the term in the instant application, it is clear that the terms "haploidentical" and "autologous" are mutually exclusive.

Turning now to the instant rejection, applicants respectfully submit that Ohno does not teach haploidentical APCs as recited in the instant claims. It is noted that the Patent Office concedes this point on page 6 of the Final Official Action by stating that "Ohno does not teach dendritic cells from haploidentical individuals" (emphasis added). In further view of the fact that autologous APCs are not HLA-haploidentical APCs and would actually be understood by one of ordinary skill in the art to be expressly distinguished from HLA-haploidentical APCs, applicants respectfully submit that Ohno does not disclose every element of independent claims 23, 27, and 33, each of which recites semi-allogeneic HLA-haploidentical APCs.

Accordingly, applicants respectfully submit that Ohno does not support a rejection of claims 23, 27, and 33 under 35 U.S.C. § 102(e). Claims 28, 30, 32, 34, 37-40, and 43-46 all depend directly or indirectly from one of claims 23, 27, and 33, and thus are also believed to be distinguished over Ohno. As a result, applicants respectfully request that the instant rejection of claims 23, 27, 28, 30, 32-34, 37-40, and 43-46 be withdrawn at this time.

IV. Responses to the Obviousness Rejections

Claims 23, 25, 27, 28, 30, 32-34, 36-40, and 43-46 have been rejected under 35 U.S.C. § 103(a) upon the contention that the claims are unpatentable over Ohno in view of Nair. Claims 23-25, 27, 28, 30, 32-34, and 36-46 have also been rejected under this section upon the contention that they are unpatentable over Ohno and Nair and further in view of Storkus. Claims 23, 27, 28, 30, 32-34, 37-40, and 43-46 have also been rejected under this section upon the contention that they are unpatentable over Ohno.

After careful consideration of the rejections and the Patent Office's bases therefor, applicants respectfully traverse the rejections and submit the following remarks.

IV.A. Response to the Rejection over Ohno in view of Nair

Claims 23, 25, 27, 28, 30, 32-34, 36-40, and 43-46 have been rejected under 35 U.S.C. § 103(a) upon the contention that the claims are unpatentable over Ohno in view of Nair. According to the Patent Office, Ohno teaches fusions of autologous dendritic cells with tumor cells for the expression of the tumor cell peptides by the dendritic cell. The Patent Office concedes, however, that Ohno does not teach autologous dendritic cells which are transfected with nucleic acids encoding tumor cell peptides to produce the expression of said peptides by the autologous dendritic cells. The deficiency is asserted to be cured by Nair, which the Patent Office contends teaches a method for loading dendritic cells by introduction of a tumor associated RNA which is unfractionated or cDNA made by PCR. From this, the Patent Office asserts that it would have been *prima facie* obvious to substitute the RNA- or cDNA-transfected dendritic cells to a patient having cancer. One of skill in the art would allegedly have been motivated to do so by the teachings of Nair regarding the improvements associated with using unfractionated RNA for the loading of dendritic cells, and the administration the loaded dendritic cells as part of the immunotherapy as taught by Nair.

Applicants respectfully submit that the combination of Ohno and Nair does not support a rejection of the pending claims under 35 U.S.C. § 103(a). Applicants respectfully submit that as discussed in more detail hereinabove with respect to the anticipation rejection, autologous dendritic cells are not semi-allogeneic HLA-

haploididentical APCs as recited in the claims, and thus the combination of Ohno and Nair fails to support the instant rejection.

Applicants further respectfully submit that although the Patent Office has not asserted this point in connection with the instant rejection, the Patent Office's contention that it would have been *prima facie* obvious to use a mixture of haploididentical dendritic cells and autologous dendritic cells in the event that there was a deficiency in the quantity of dendritic cells obtained from the patient finds no support in any reference on the record, and thus is improper. Specifically, there is no disclosure in either Ohno or Nair that the quantity of dendritic cells obtainable from a subject would ever be insufficient. Therefore, the Patent Office's attempt to integrate haploididentical APCs into the disclosure of Ohno can only be based on an impermissible hindsight reconstruction of Ohno, particularly in view of the fact that Ohno itself describes several strategies for purifying and/or generating as well as enriching for dendritic cells (see Ohno at paragraphs [0074]-[0079]).

Summarily, applicants respectfully submit that even if one of ordinary skill in the art were to combine Ohno and Nair as suggested by the Patent Office, he or she would not arrive at the subject matter of the instant claims: semi-allogenic, HLA-haploididentical (*i.e.*, non-autologous) APCs. As such, applicants respectfully submit that the combination of Ohno and Nair does not support a rejection of claims 23, 25, 27, 28, 30, 32-34, 36-40, and 43-46 under 35 U.S.C. § 103(a).

I.V.B. Response to the Rejection over Ohno in view of Nair  
and further in view of Storkus

Claims 23-25, 27, 28, 30, 32-34, and 36-46 have also been rejected under this section upon the contention that they are unpatentable over Ohno and Nair and further in view of Storkus. According to the Patent Office, Storkus teaches that dendritic cells can be pulsed with HLA-attached allogeneic tumor cell lines as an alternative to acid eluted peptides from the patients tumor cells, the administration of pulsed dendritic cells by intravenous routes, and that the invention can be applied to treat colon, squamous, gastric, breast, prostate, lung, cervical and ovarian carcinomas.

Applicants respectfully submit that the combination of Ohno, Nair, and Storkus does not support a rejection of the pending claims under 35 U.S.C. § 103(a).

Particularly, applicants respectfully submit that Storkus does not cure the deficiencies of Ohno and Nair set forth hereinabove.

Accordingly, applicants respectfully submit that the Patent Office has not presented a *prima facie* case of obviousness of claims 23-25, 27, 28, 30, 32-34, and 36-46 over Ohno and Nair and further in view of Storkus. As such, applicants respectfully request that the instant rejection be withdrawn at this time.

*IV.C. Response to the Rejection over Ohno*

Claims 23, 27, 28, 30, 32-34, 37-40, and 43-46 have also been rejected under this section upon the contention that they are unpatentable over Ohno. According to the Patent Office, Ohno teaches using antigen-presenting cells that are autologous.

Applicants respectfully submit that Ohno does not support the instant rejection for the reasons set forth hereinabove with respect to the previous rejections. In fact, in the context of the instant rejection, the Patent Office concedes that Ohno does not teach dendritic cells from haploidentical individuals. However, the Patent Office asserts that it would have been *prima facie* obvious to use a mixture of haploidentical dendritic cells and autologous dendritic cells in the event that there was a deficiency in the quantity of dendritic cells obtained from the patient, and that one of skill in the art would have been motivated to provide more dendritic cells in place of the autologous dendritic cells in order to obtain enough of the dendritic cell-tumor cell chimeric cells with which to treat the patient.

Applicants respectfully submit that the Patent Office has provided no support for the contention that one of ordinary skill in the art would have been motivated to use a mixture of haploidentical dendritic cells and autologous dendritic cells. The Patent Office merely speculates that a situation might arise in which the quantity of autologous dendritic cells isolated from the patient would be inadequate. Applicants respectfully submit that there is no basis for this speculation, as Ohno itself teaches methods for isolating and/or generating as well as enriching dendritic cells from subjects (see Ohno at paragraphs [0074]-[0079]).

As a result, applicants respectfully submit that it is clear that the Patent Office has employed impermissible hindsight in an attempt to find a rationale for including non-

autologous dendritic cells in the method of Ohno since there is no suggestion in the reference that the need would ever arise.

Accordingly, applicants respectfully submit that the Patent Office has not presented a *prima facie* case of obviousness of claims 23, 27, 28, 30, 32-34, 37-40, and 43-46 over Ohno. As a result, applicants respectfully request that the instant rejection be withdrawn at this time.

#### CONCLUSION

In light of the above, it is respectfully submitted that the present application is now in proper condition for allowance, and a Notice of Allowance to that effect is earnestly solicited.

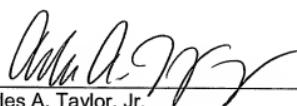
If any small matter should remain outstanding after the Patent Examiner has had an opportunity to review the above Remarks, the Patent Examiner is respectfully requested to telephone the undersigned patent attorney in order to resolve these matters and avoid the issuance of another Official Action.

#### DEPOSIT ACCOUNT

The Commissioner is hereby authorized to charge any fees associated with the filing of this correspondence to Deposit Account No. 50-0426.

Respectfully submitted,  
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## EXHIBIT A

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are reluctant to take this step, which frustrates the attainment of an objective they themselves desire.

This small series is reported because we believe that jaw wiring followed by the fitting of a waist cord could be a valuable treatment for severe obesity. Unlike the bypass procedures it is inexpensive and safe. Only one of our patients (case 4, table I) seems unwilling to tolerate the waist cord: the others are maintaining their weight at a level just below that at which it is uncomfortable. Several patients have volunteered the observation that they would have gained weight on holiday had it not been for the waist cord, and they are anxious that it should not be removed. This result supports the hypothesis that such factors as the tightness of clothing are important in regulating body weight.<sup>12</sup>

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## Treatment of severe aplastic anaemia with antilymphocyte globulin or bone-marrow transplantation

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## Abstract

Fifty-three patients with severe aplastic anaemia were admitted to this hospital between January 1976 and June 1980, of whom three arrived in terminal condition and died before treatment for their basic disease could be given. Thus 50 patients were treated and evaluated in a prospective study according to one protocol. Eighteen patients with an HLA-identical sibling underwent bone-marrow transplantation with the aim of achieving haematopoietic chimerism. Thirty-two patients without an HLA-identical sibling were given antilymphocyte globulin with or without an infusion of HLA-haplotypoid marrow. All these 32 patients received low-dose androgens after the procedure. In the first group eight

patients (44%) survived. In the two other groups, 22 patients survived (69%), of whom 20 were completely self-sustaining (63%). Engraftment and graft-versus-host disease did not occur in the group who received antilymphocyte globulin and haploidoid marrow, and the haematopoietic reconstitutions in these patients were all autologous.

These results confirm the efficacy of antilymphocyte globulin in the treatment of severe aplastic anaemia and show that such treatment is at least as good as bone-marrow transplantation. Its mechanism of action remains unknown, but most patients with aplastic anaemia have a pool of haematopoietic stem cells able to repopulate the marrow after this type of treatment.

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## Introduction

The potential usefulness of antilymphocyte globulin followed by allogeneic marrow infusion in treating aplastic anaemia was first reported in 1970.<sup>1</sup> In 1977 we reported the results in 29 patients with severe aplastic anaemia treated with antilymphocyte globulin with or without marrow infusion.<sup>2</sup> The rationale<sup>3</sup> and experimental<sup>4</sup> and clinical<sup>5</sup> basis for this therapeutic approach to aplastic anaemia have been outlined. Despite these early results doubts remained about the usefulness of antilymphocyte globulin because of possible patient selection. We therefore began a study in 1976 to compare prospectively bone-marrow transplantation and treatment with antilymphocyte globulin alone or followed by bone-marrow infusion. All admitted patients with severe aplastic anaemia without exception were treated according to one protocol. They were classified

## EXHIBIT A

into three groups depending on the availability and histocompatibility of a family donor. All three groups were comparable in age, transfusion history, severity of pancytopenia, interval between diagnosis and treatment, previous treatment with androgens, and all factors known to influence the prognosis of severe aplastic anaemia and the fate of haematopoietic grafts.<sup>7-9</sup> We report here the results obtained.

## Patients and methods

We studied all the patients referred to Kantonsspital Basle between January 1976 and June 1980. All had severe aplastic anaemia and fulfilled at least two of the three blood criteria and the criterion for bone-marrow histology.<sup>4</sup> They were classified on admission according to the criteria shown in table I. All patients surviving the time required for histocompatibility testing were treated. Most of them had been referred from other medical centres, where conventional treatment has been tried, and so the interval between diagnosis and treatment varied widely.

All three groups were comparable in age, aetiology, previous treatment, and blood counts (table II). They were also comparable in their transfusion history, but in most of the referred patients exact data were difficult to obtain. Three patients had not received transfusions (one in each group), while the others had received many transfusions and depended on regular substitution of blood components. Three patients (one in each group) had a long history of aplastic anaemia. This had remitted initially but they were sent to us in relapse. Three patients were in terminal stage on arrival and died despite maximal

support before treatment could be given. The remaining 50 patients were allocated to one of three groups.

**Group A** comprised 18 patients (nine male, nine female) who had an HLA-identical sibling donor. They were all given four doses of 50 mg cyclophosphamide/kg followed by a marrow graft consisting of a median of  $3.75 \times 10^8$  (range 2.9-13.0  $\times 10^8$ ) nucleated marrow cells/kg. The median age was 18 (range 4-29) years. In seven cases there was a major ABO barrier that required plasma exchange. The initial seven patients were given only marrow. The remaining 11 were given unirradiated peripheral blood buffy coat from four units of blood from the marrow donor on each of five successive days after bone-marrow transplantation. The first 14 patients were given methotrexate<sup>10</sup> for prophylaxis against graft-versus-host disease, while the last four were given cyclosporin A.<sup>11</sup>

**Group B** comprised 20 patients (16 male, four female) who had no HLA-identical sibling donor but for whom an HLA-haplotypoid, ABO-compatible family donor was available. They were given antilymphocyte globulin 40 mg/kg on each of four successive days followed by a marrow infusion consisting of a median of  $2.3 \times 10^8$  (range 1.2-5.5  $\times 10^8$ ) nucleated marrow cells/kg. This was followed by oral norethandrolone 0.5-1.0 mg/kg/day for four to six months, after which the dose was gradually reduced. The median age was 22 (range 10-49) years.

**Group C** comprised 12 patients (seven male, five female) who had no family donor available, preformed antibodies against all potential donors, or major ABO barriers or whose potential donor was ill and at high risk for anaesthesia. They were treated with four doses of antilymphocyte globulin 40 mg/kg alone followed by norethandrolone, as in group B. The median age was 23 (range 7-37) years.

*Antilymphocyte globulin*—Equine antilymphocyte globulin from the same horse was used throughout the study (Schweiz Serum- und Impfhaus, Berne). The horse was immunised every six weeks by human thoracic-duct lymphocytes. In patients who relapsed rabbit antilymphocyte globulin RIV (Holland) was given. Both preparations prolong skin allograft survival in rhesus monkeys.

TABLE I—Classification criteria and treatment

Group	Criteria	Treatment
A	HLA-A, B, Dr identical sibling donor, mixed leucocyte culture not reactive	Cyclophosphamide 50 mg/kg $\times 4$ + bone-marrow transplantation
B	HLA-haplotypoid, ABO-identical, cross-match negative family donor	Antilymphocyte globulin 40 mg/kg $\times 4$ + bone-marrow infusion Norethandrolone 0.5-1 mg/kg/day by mouth
C	No donor	Antilymphocyte globulin 40 mg/kg $\times 4$ Norethandrolone 0.5-1 mg/kg/day by mouth

TABLE II—Clinical details of patients in the three groups

Group	No in group	Median age (years) (and range)	Sex	Aetiology	Median interval between diagnosis and treatment (months) (and range)	No receiving androgens	Median blood counts on admission ( $\times 10^9/l$ ) (and range)		
							Retieutocytes	Granulocytes	Thrombocytes
A	18	18 (4-29)	9M, 9F	15 idiopathic 2 posthepatitis 2 aplastic (1 T) 1 benzene (1 amiodarone)	3.5 (1-72)	9	4.4 (0-100)	0.2 (0-19)	3 (1-25)
B	20	22 (10-49)	16M, 4F	14 idiopathic 3 aplastic (1 chloramphenicol, 1 benzene, 1 amiodarone) 2 posthepatitis 1 aplastic 1 toxic (1 chloramphenicol, 1 benzene)	6 (1-28)	11	8.9 (0-38)	0.25 (0-1.2)	6 (0-16)
C	12	23 (7-37)	7M, 5F	10 idiopathic 2 toxic (1 chloramphenicol, 1 benzene)	5 (1-168)	6	5 (0-67)	0.15 (0-6)	7.5 (2-20)

TABLE III—Results of treatment

Group	No of patients	No of deaths	Cause of death	No of survivors	State of survivors
A	18	10	3 rejection 5 graft-versus-host disease + interstitial pneumonia 2 aplastic 2 sepsis, intracranial haemorrhage	8 (44%)	5 chimeras alive and well 5 with autologous reconstitution alive and well 2 with chronic graft-versus-host disease
B	20*	5	2 rejection, intracranial haemorrhage 2 intracranial haemorrhage after bone-marrow infusion 1 aplastic 1 loss to follow-up (not evaluable)	14 (74%)	12 chimeras alive and well 1 needing occasional transfusions of red blood cells
C	12	4	2 intracranial haemorrhage 2 aplastic 1 graft-versus-host disease after bone-marrow transplantation	8 (67%)	7 alive and well† 1 needing transfusions of red blood cells and platelets

\* Nineteen were evaluable as one was lost to follow-up.

† One after antilymphocyte globulin and haplotypoid marrow.

haematopoietic chimeras and one had complete autologous haematopoietic reconstitution. Of the 10 deaths, four were caused by rejection and infection and six by graft-versus-host disease and interstitial pneumonia. Of the first seven patients, who received only marrow, three survived, two as chimeras and one with autologous reconstitution. Of the four deaths, three were due to rejection and infection and one to graft-versus-host disease and interstitial pneumonia. All 11 patients given marrow and buffy-coat infusion showed prompt engraftment. One died early from infection. Six patients given prophylactic methotrexate developed moderate to severe graft-versus-host disease, which caused the death of four in conjunction with

responder an HLA-identical sibling donor became available one year after the initial treatment. She received a graft but died from chronic graft-versus-host disease and infection. Thus of the four deaths in this group, three were caused by cerebral haemorrhage due to alloimmunisation with refractoriness to all available platelet support and one by graft-versus-host disease after an allogeneic marrow graft.

## Discussion

Despite optimal supportive care, the outlook for patients with severe aplastic anaemia who receive conventional treatment is still poor. In a large prospective study<sup>4</sup> only 25% of the patients survived regardless of whether they were given androgens but 57% of the patients treated with bone-marrow transplantation survived. Many workers in the field therefore think that treatments aiming at complete haematopoietic chimerism are the only rational approach to severe aplastic anaemia.<sup>11</sup> Even with an HLA-identical sibling, however, results need to be improved. In the most recent multicentre study, using this approach in 144 patients, one-year survival was 44%.<sup>9</sup> This is identical with the results achieved in our group of patients, in whom the aim was haematopoietic chimerism. These results are not optimal, and in addition it must be remembered that 10-20% of the surviving patients suffer from chronic graft-versus-host disease. Much hope came from the initial studies with total lymphoid irradiation used in conjunction with cyclophosphamide for conditioning,<sup>12</sup> which yielded high engraftment rates and a low incidence of graft-versus-host disease. These data came from children, however, and could not be confirmed in adults.<sup>13</sup>

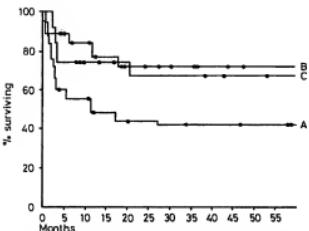


FIG 1.—Comparison of survival in groups A, B, and C.  
●—Surviving patient.

TABLE IV.—Median blood counts (and ranges) ( $\times 10^9/l$ ) in surviving patients at last measurement made during follow-up

Group	No surviving	No self-sustaining	Hæmoglobin (g/dl)	Reticulocytes	Granulocytes	Thrombocytes
A	8	8	13.7 <sup>a</sup> (10.3-15.9)	29 (22-98)	4 (2.1-7.3)	103-283
B	14	13	14.3 <sup>a</sup> (8.4-15.9)	47.5 (19-172.0)	1.7 (0.5-3.3)	90 (1.0-340)
C	8	7	10.5 <sup>a</sup> (8.2-15.6)	54 (6-70)	1.9 (0.9-2.2)	40 (9-190)

interstitial pneumonia or overwhelming infection. The two survivors suffered from disabling chronic graft-versus-host disease. Of the four patients given cyclosporin A instead of methotrexate as prophylaxis against graft-versus-host disease, three survived; one patient died from generalised cytomegalovirus infection while the others remained alive and well without signs of graft-versus-host disease.

**Group A** ( $n=20$ )—One patient was lost to follow-up at two months, but of the 19 evaluable patients, 14 survived (74%). Thirteen of these had completely self-sustaining haematopoiesis, one needed occasional red-cell transfusions. All continued taking low-dose androgens (noretandrolone 5-10 mg/day). They were all leading a normal life and there were no chronic problems. Five patients died, two from massive intracranial haemorrhage 24-48 hours after marrow infusion. One showed no response and died from an acute septic infection in another hospital. Three of the initial responders relapsed after stopping the androgens and were given rabbit antithymocyte globulin. One went into complete remission again, but two died from cerebral haemorrhage due to alloimmunisation and complete refractoriness to all platelet donors that were available.

**Group C** ( $n=12$ )—Eight patients survived (67%), seven with self-sustaining haematopoiesis. All continued to receive androgens (noretandrolone 5-10 mg/day). One relapsed but responded to a second course of treatment with rabbit antithymocyte globulin. A second relapse occurred, and this patient then remained transfusion dependent. Two patients showed no response to antithymocyte globulin alone but remained alive. One of them was treated with rabbit antithymocyte globulin followed by infusion of HLA-haplotypoid marrow three years later. He responded with complete haematopoietic recovery and was subsequently treated by phlebotomies for iron overload from previous transfusions. For the other non-

Virtually all patients have received many transfusions when they are referred to us. Such sensitisation seems to be the most common cause of graft rejection.<sup>1</sup> If patients receive a transplant before they need transfusions results are significantly better.<sup>14</sup> New hope for preventing graft-versus-host disease comes now from prophylactic cyclosporin A. In the four patients in whom we used this drug instead of methotrexate, no severe graft-versus-host disease was seen. Because of the small number of patients, however, conclusive statements are impossible. Nevertheless, with our experience with bone-marrow transplantation in acute leukaemia, when we saw no severe graft-versus-host disease in 10 successive patients treated with cyclosporin A, we think that this is a promising approach to preventing such disease.<sup>15</sup> The value of the drug in bone-marrow transplantation using HLA non-identical donors has not yet been established.

The results achieved in groups B and C support further our assumption that in most cases the pathogenetic mechanism in severe aplastic anaemia is a disturbed maturation in the haematopoietic precursor cell compartment rather than a defect at the level of the pluripotent stem cell itself.<sup>5</sup> The autologous reconstructions seen after treatment with antithymocyte globulin and androgens with or without marrow infusion are usually less complete than haematopoietic chimerism. In particular, median platelet and granulocyte counts are lower than in chimeras. Furthermore, it takes much longer for these patients to become self-sustaining and independent of transfusions (fig 2). Another concern is androgen dependence. Until 1978 we completely

## EXHIBIT A

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stopped androgens as soon as remission was reached but saw three relapses within two months. One patient responded to rabbit antithymocyte globulin and was in complete remission taking continuous androgens one year after the second treatment. Two patients died from cerebral haemorrhage after antithymocyte globulin due to severe alloimmunisation and refractoriness to all available platelet donors. Subsequently we kept our patients on low-dose androgens—that is, norethandrolone 10 mg daily or, later, every other day. With this dose we did not see any serious side effects or relapses. Intriguingly, even in complete haematological remission in-vitro growth of haematopoietic precursor cells (BFU-E, CFU-E, and GM-CFU-C) is appreciably decreased.<sup>14</sup> Adding autologous peripheral mononuclear cells to the marrow cultures has a strong inhibiting effect.<sup>15</sup> This phenomenon potentially indicates latent disease. Many of our patients remained in complete autologous remission for four to over seven years, so this laboratory phenomenon may not necessarily imply that they still have life-threatening disease.

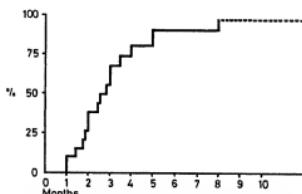


FIG 2—Time required in groups B and C ( $n=13$  and  $n=6$  respectively) until patients self-sustaining and transfusion independent.

The role of marrow infusion was obvious in experimental benzene aplasia in rabbits where antilymphocytic serum alone was ineffective.<sup>3</sup> Clinically, there was no significant difference between groups B and C, and we do not know whether the marrow infusion added anything to the final result. Two patients died within 24–28 hours after marrow infusion from massive intracranial haemorrhage. Both had pronounced thrombocytopenia at the time of infusion. The most likely explanation for this phenomenon is that the heparin in the marrow might play a role in initiating haemorrhage. In the last 10 patients we therefore gave protamine sulphate immediately after the marrow to neutralise the heparin.

This prospective study of patients with severe aplastic anaemia has not solved all the questions about the pathogenesis and treatment of the disease. Nevertheless, it shows clearly that most of these patients have enough residual stem cells to repopulate the marrow and lead to haematopoietic remissions. This is extremely important for patients without an HLA-identical or syngeneic sibling donor. At present we cannot say whether antilymphocyte globulin really abrogates an autoimmune process in the marrow or makes stem cells sensitive to androgens in a non-specific way. Marrow infusion without the aim of engraftment has an experimental basis.<sup>16</sup> We have continued to use it clinically because in our experience remissions have appeared to be more complete than with antilymphocyte globulins and androgens alone. Furthermore, one patient who did not respond to antilymphocyte globulin and androgens went into complete remission after HLA-haploidentical marrow was given in addition.

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(Accepted 18 December 1980)

THE BRAMBLE, OR BLACK-BERRY BUSH. It is so well known that it needs no description. The virtues thereof are as follows:

It is a plant of Venus in Aries. If any ask the reason why Venus is so prickly? Tell them it is because she is in the house of Mars. The buds, leaves, and branches, while they are green, are of a good use in the ulcers and purid sores of the mouth and throat, and of the quinsy, and likewise to heal other fresh wounds and sores; but the flowers and fruit unripe are very binding, and so profitable for the bloody flux, lasks, and are a fit remedy for spitting of blood. Either the decoction of the powder or the root taken, is good to break or drive forth gravel and the stone in the reins and kidneys. The leaves and stalks, as well green as dry, are exceeding good lotions for sores in the mouth, or other secret parts. The decoction of them, and of the dried branches, do much bind the belly and are good for too much flowing of women's courses; the berries of the flowers a powerful remedy against the poison of the most venomous serpent; as well drunk as outwardly applied, helps the sores of the fundament and the piles; the juice of the berries mixed with the juice of mulberries, do bind more effectually, and helps all fretting and eating sores and ulcers wheresoever. The distilled water of the branches, leaves, and flowers, or of the fruit, is very pleasant in taste, and very effectual in fevers and hot distempers of the body, head, eyes, and other parts, and for the purposes aforesaid. The leaves boiled in lye, and the head washed therewith, heals the itch and running sores thereof, and makes the hair black. The powder of the leaves strewed on cankers and running ulcers, wonderfully helps to heal them. Some use to condense the juice of the leaves, and some the juice of the berries, to keep for their use all the year, for the purposes aforesaid. (Nicholas Culpeper (1616-54) *The Complete Herbal*, 1850.)

## EXHIBIT B

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# Autologous bone marrow mixed with HLA-haploidentical allogeneic marrow transplantation for treatment of patients with malignant blood diseases

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### Summary:

We have previously demonstrated that syngeneic marrow mixed with H-2 haploidentical marrow transplantation could provide not only protection against graft-versus-host disease (GVHD) but also anti-leukemic (GVL) effects in mice. In the present studies, we report clinical observations using autologous marrow mixed with HLA-haploidentical allogeneic marrow transplantation for treatment of patients with malignant blood diseases. Sixteen cases, including 12 with acute leukemia and four with advanced malignant lymphoma, were treated by autologous marrow, which was purged *in vitro* by hyperthermia (42.5°C for 70 min) following incubation for 5 days with interleukin 2 (IL-2) in liquid culture and mixed with HLA haploidentical marrow cells from their sibling or parent. Acute GVHD was not observed in any patient after transplantation. Hematological rescue in the clinical setting was demonstrated in all cases but one who died early from hepatic veno-occlusive disease (VOD). Five cases who were transplanted at the time of CR2 or CR3 and in advanced phase of lymphoma, relapsed 4 to 7 months after transplantation. The relapse rate was 31.3%. None of eight patients who received allogeneic BMT within 2 h after ABMT relapsed with median follow-up of 12 months and two of them died from procedure-related complications. Seven cases are still alive and disease-free with a median follow-up of 12 months. Mixed chimerism was found in 3/6 cases, who had different sex donors, by analysis of sex chromosomes. These results show that mixed transplantation is a safe, effective and new approach to treat patients with malignant tumors. In order to detect the effects of GVL, studies are now in progress in our clinic.

**Keywords:** bone marrow transplantation; mixed graft-versus-leukemia; malignant blood diseases

Bone marrow transplantation is essentially hematopoietic stem cell transplantation (HSCT). With increasing knowledge of the nature of the stem cell, the characterization of stem cell regulation, especially the interaction of the hematopoietic cells with the microenvironment of the marrow, it has been applied to an increasingly wide spectrum of diseases. More than 10 000 transplants are being done every year throughout the world, including allogeneic bone marrow transplantation (allo-BMT), and autologous bone marrow transplantation (ABMT) or autologous peripheral blood stem cell transplantation (ABSCT).<sup>1,2</sup> Interdisciplinary research by numerous investigators around the world continues to expand the scientific basis and clinical results of marrow transplantation. However, for treatment of malignant blood diseases, particularly leukemia, allo-BMT and ABMT have their strong points and weaknesses, respectively.<sup>3,4</sup> The major advantage of allo-BMT is lower relapse after grafting, probably due to graft-versus-leukemia (GVL) effects mediated by alloreactive donor lymphocytes. Unfortunately, its disadvantage is also obvious. First, it is limited to a select group of patients with an HLA-identical sibling as the donor; only about 40% of patients with acute leukemia have histocompatible donors in western countries. It will be difficult to find donors in China in the future, because of birth control. Secondly, it is generally limited to patients under the age of 45 to 50 years. Finally, it is associated with a variety of potentially lethal complications, particularly graft-versus-host disease (GVHD) and interstitial pneumonitis, which results in higher graft-related mortality. Therefore, ABMT has been widely applied in clinics. It is donor-unlimited, has fewer complications, and the patient's age is relatively wider, but it has a high relapse risk after transplantation.<sup>4</sup> If ABMT mixed with allo-BMT could be used, in order to make full use of advantages in both ABMT and allo-BMT and overcome their shortcomings, it would be a new approach to treating malignant diseases.

In the present studies, we report clinical observations using autologous marrow mixed with HLA-haploidentical allogeneic marrow transplantation (MBMT) in the successful treatment of patients with malignant blood diseases.

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## EXHIBIT B



### Patients and methods

#### Patient characteristics

From February 1993 to May 1996 16 patients with malignant blood diseases underwent MBMT. Of these, 12 had acute leukemia (ALL five patients, ANLL seven patients), four advanced malignant lymphoma (Hodgkin's lymphoma one patient, non-Hodgkin's lymphoma three patients). The median age at the time of transplantation was 25 years (range 21–49). The clinical characteristics of the patients are summarized in Table 1. At the time of MBMT, six patients were in first complete remission (CRI), eight in CR2 and two in CR3.

#### Informed consent

Informed consent for marrow harvesting and MBMT was obtained in all patients and donors.

#### Bone marrow cell collection, processing and purging *in vitro*

Marrow cell harvest, processing, and purging *in vitro* have been described previously.<sup>5</sup> Briefly, marrow cells were obtained by multiple aspirations from the posterior and, if necessary, the anterior iliac crests, until the desired total number of cells was collected. The mononuclear cells (MNCs) were separated by sedimentation supplemented with 0.4% methylcellulose at 4°C for about 2 h.<sup>6</sup> The MNCs were purged by hyperthermia (42.5°C for 70 min) *in vitro* at first,<sup>7</sup> then suspended in the liquid culture system of Iscove's medium (Gibco, Paisley, UK) at a concentration of 1–2 × 10<sup>7</sup>/ml, which was supplemented with 25% autologous serum, penicillin 100 units/ml and gentamicin

40 mg/ml and cultured in a 175 cm<sup>2</sup> tissue culture flask. Human recombinant IL-2 (Sinochem United Cross Pharm, Beijing, China) was added at the beginning of the culture at a concentration of 1000 units/ml.<sup>8</sup> All flasks were placed in a humidified incubator with 5% CO<sub>2</sub> and maintained at 37°C for 5 days. Supernatant cells and adherent cells were harvested and washed in 4% human serum albumin solution. The sterility of autografts after culture was routinely confirmed by cultures for bacteria and fungi.

#### Conditioning regimen

The ablative-dose chemoradiotherapy consisted of vincristine (VCR), cytosine arabinoside (Ara-C), lomustine (CCNU) or VP16, mitoxantrone and cyclophosphamide (CP) plus total body irradiation (Hd-VAC(E)MC+TBI) as a conditioning regimen in preparation for ABMT.<sup>9</sup> The Hd-VAC(E)MC+TBI regimen is shown in Table 2 in detail. In lymphoma patients, boost irradiations (total dose 10–12 Gy) were given over the bulky and original tumor areas.

Table 2 Hd-VAC(E)MC+TBI conditioning regimens

Day	Contents
-5	Bone marrow harvesting, then TBI 6–7 Gy, followed by VCR 2 mg i.v.; CCNU 200 mg oral, or VP16 200 mg i.v.; Ara-C 750–1000 mg i.v.
-4	Ara-C 750–1000 mg i.v.; Mit 10 mg i.v.
-3	Mit 10 mg i.v.
-2	CP 60 mg/kg i.v.
-1	rest
0	Autologous bone marrow reinfusion followed HLA-haploid-identical allogeneic marrow transplantation

Table 1 Clinical data of 16 patients

UPN	Sex/Age	Diagnosis	State at MBMT	No. of autograft cells		Donors		Blood type		Current status or cause of death
				MNC (×10 <sup>8</sup> /kg)	GM-CFU (×10 <sup>3</sup> /kg)	Sex	Graft MNC (×10 <sup>8</sup> /kg)	GM- CFU (×10 <sup>3</sup> /kg)	Recp	
1	M/21	ANLL-M4	CR2	0.41	0.12	M	0.07	0.11	O	O CRR 40 months+
2	M/22	ALL-L2	CR1	0.61	0.23	F	0.11	0.023	O	A 7m relapse, 8m died
3	F/25	ANLL-M4	CR3	0.66	0.23	M	0.11	—	B	B 4m relapse, 5m died
4	F/25	ALL-L3	CR3	0.67	0.23	M	0.11	0.08	O	O 3 weeks died from HVOD
5	M/21	ALL-L2	CR1	0.83	—	F	0.14	—	AB	A 4m relapse, 5m died
6	F/22	ALL-L1	CR2	0.95	1.15	M	0.13	—	A	A 7m relapse, 14m died
7	M/31	ALL-L2	CR2	0.73	1.16	M	0.12	0.18	AB	AB 7m died from encephalopathy
8	F/41	HL,LD,IVB	CR2	0.19	0.3	M	0.19	0.3	O	B 4m relapse, 4.5m died
9	F/29	NHL,HR,IIB	CR1	0.52	3.92	M	0.09	0.35	A	A CCR 16 months+
10	M/22	HL,NS,IVB	CR2	0.56	1.23	M	0.09	0.56	O	A 8 months died from fulminant hepatitis
11	M/21	ANLL-M5	CR2	0.57	2.33	F	0.10	1.34	A	A 3 months died from appendicular perforation
12	M/23	ANLL-M3	CR1	0.53	2.55	M	0.09	0.80	AB	A CCR 15 months+
13	M/36	ANLL-M4	CR1	0.65	1.94	F	0.11	0.57	O	A CCR 12 months+
14	M/32	HL,Mix,IIB	CR2	0.32	1.87	F	0.05	0.38	O	O CCR 12 months+
15	M/41	ANLL-M4	CR1	0.65	2.20	F	0.11	0.41	A	A CCR 9 months+
16	F/25	ANLL-M3v	CR2	0.60	2.75	M	0.10	0.40	B	B CCR 6 months+

LC = lymphocyte depleted; NS = nodular sclerosis; Mix = mixed cellularity; HR = high grade including large cell, immunoblastic or lymphoblastic and small noncleaved cell; CCR = continuous CR, m = month, + = still survive; UPN = unique patient number.

## EXHIBIT B

Mixed transplantation for malignant diseases  
WM Da et al



### *Donor selection*

Selection of a donor was based on HLA-A,B,C typing performed by complement-dependent cytotoxicity. All the donors were HLA-haploidentical matched. Twelve were siblings and four were parents. Among them, the major ABO incompatibility between recipient and donor was five pairs. For them, the donor's marrow cells were transplanted after removing the RBC using separation of sedimentation with 0.4% methylcellulose at 4°C.

### *GM-CFU and CFU-L assay*

The assay for GM-CFU was performed according to the method of Pike and Robinson.<sup>7</sup> One milliliter of 0.3% agar in RPMI-1640 medium supplemented with 20% fetal calf serum (FCS) and 20% human placenta-conditioned medium containing  $2 \times 10^5$  MNC was cultured in triplicate samples for 9 days and scored for colonies (>40 cells). The result used was the mean value of the triplicate reading. The assay for CFU-L was based on a modified method described by Buick et al.<sup>7</sup> Briefly, 1 ml of 0.3% agar in Iscove's medium supplemented with 20% FCS and 15% phytohemagglutinin (PHA) leukocyte-conditioned medium containing  $2 \times 10^5$  MNC was plated into 35-mm glass dishes and cultured in humidified 5% CO<sub>2</sub>, 95% air at 37°C. All cultures were done in triplicate. After 7 days of incubation, colonies containing more than 20 cells were counted. CFU-L were defined by morphological examination of isolated individual colonies.

### *Mixed transplantation*

In the first eight patients, allogeneic bone marrow cells were reinfused 6 h after ABMT (group 1), and the latter eight patients, 2 h after ABMT (group 2). The mean autologous marrow MNCs and GM-CFU transplanted were  $0.54 \times 10^8$ /kg, and  $1.47 \times 10^4$ /kg, respectively. According to the result in mice,<sup>16</sup> the number of donor's marrow MNCs and GM-CFU transplanted were  $0.096 \times 10^8$ /kg and  $0.41 \times 10^4$ /kg, which were approximately a sixth and a fourth of autologous grafting, respectively (Table 3).

All patients were managed in laminar flow isolation with gut decontamination until their white blood cells (WBC) reached  $2 \times 10^9/l$ . During the period of marrow aplasia, antibiotics, platelet components, and other supportive care were also given.

### *Application of cytokines*

In order to protect against GVHD, and enhance anti-leukemic effects (GVL), all patients were treated with human

Table 3 Mixed cells grafted

	ABMT	Donor
MNCs ( $\times 10^8/kg$ )	0.536	0.096
GM-CFU ( $\times 10^4/kg$ )	1.57	0.41
L-CFU ( $\times 10^4/kg$ )	0.004	0

Table 4 Median recovery after manipulation

	No. of cases	Before	After
MNCs ( $\times 10^8/kg$ )	16	1.0	0.55
GM-CFU ( $\times 10^4/kg$ )	16	4.04	1.43
L-CFU ( $\times 10^4/kg$ )	16	0.18	0.004
Volume (ml)	16	1172	460

recombinant interleukin 2.<sup>10-12</sup> The starting dose was  $100 \times 10^4$  unit/day for 4 days,  $20 \times 10^4$  unit/day for 1 week. We also used the human recombinant granulocyte colony-stimulating factor (rhG-CSF, Filgrastim; Kirin, Japan),  $3 \times 10^6$  unit/day, i.v. for 2 or 3 weeks after MBMT.

### *Results*

#### *BM treatment*

Initially, a median of  $1.0 \times 10^8$  (range 0.6–1.8) MNCs/kg and  $4.04 \times 10^4$  (range 2.1–6.8) GM-CFU/kg was treated with hyperthermia, and placed in culture and activated by IL-2. Treatment of the marrow resulted in a median MNCs recovery of 55% (range 28.4–80%), and GM-CFU recovery of 36.4% (range 22–67.8%). For the ANLL, the CFU-L were considerably reduced, with a median recovery of 2.2% (range 0–4.2%) (Table 4).

#### *GVHD complications*

Acute GVHD was not observed in any patients (group 1 and 2) after MBMT. However, chronic GVHD (cGVHD) occurred in eight patients in group 2, with a median of 25 days (range 21–32) post-MBMT. The major manifestations were skin lesions including thickened skin, diffuse hyperpigmentation, buccal mucositis, weight loss, chronic liver disorders, failure to thrive, and so on. The characteristics of the cGVHD in the eight cases are shown in Table 5.

Table 5 cGVHD in group 2

No. of case	Manifestation of cGVHD						Chimerism (month)
	S	M	H	L	W	F	
9	+	+	+	-	+	-	
10	+	+	+	+	+	-	
11	++	+	++	-	+	-	
12	+	+	+	+	+	+	
13	++	+	++	++	++	+	12+
14	+	+	++	+	+	-	12+
15	++	++	++	+	++	+	9+
16	+	++	++	-	+	+	-

S = skin; M = mucositis; H = cytopenia; L = liver; W = weight loss; F = fever.



## EXHIBIT B

## Survival

In terms of overall survival, five of 16 patients relapsed within 4 to 7 months after MBMT, a relapse rate of 31.3%. Four patients died from complications and non-hematologic disorders, the actuarial disease-free survival (DFS) was 44.8% with median follow-up of 12 months. However, in group 2, none of them relapsed with median follow-up of 12 months (range 6–19 months). All patients but two, who died from fulminant hepatitis and appendicular perforation at 3 and 8 months after MBMT, respectively, are still alive in DFS. In contrast, five of eight patients in group 1 relapsed with a median of 4 months, and died within 5–14 months, two died from hepatic veno-occlusive disease (HVOD) and encephalopathy at 3 and 7 weeks, respectively. Only one patient is still DFS for more than 40 months.

Figure 1 shows that overall probability of relapse-free survival (RFS) is 50.6%. The probability of RFS is 19% for group 1 and 100% for group 2 at 14 months.

## Engraftment and hematopoietic reconstitution

Fifteen patients were successfully engrafted after MBMT. The mean time for reaching a neutrophil count  $>0.5 \times 10^9/l$  was  $27(\pm 7.3)$  days, and recovery to platelets  $>50 \times 10^9/l$  was  $38(\pm 18)$  days. One patient had persistent pancytopenia for 8 months after increasing leukocytes and platelets in the short period following MBMT for cGVHD. One patient died from HVOD before complete hematologic reconstitution could occur. We did not find significant correlations between the number of GM-CFU infused and the time to reach a granulocyte count  $>0.5 \times 10^9/l$  or platelet count  $>50 \times 10^9/l$ .

## Chimerism in survivors

Mixed chimerism was found in three of six cases, who had different sex donors, by analysis of sex chromosomes. Two of them have been followed-up for more than 12 months.

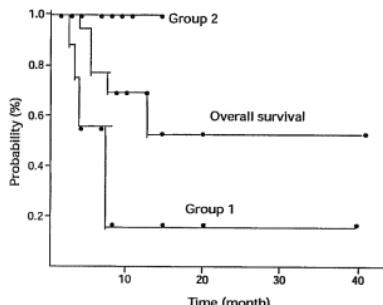


Figure 1 Relapse-free survival after MBMT.

## Discussion

In order to exploit the advantages of allogeneic and autologous hematopoietic stem cell transplantation, and to develop a new effective approach for treating patients with malignant diseases, particularly malignant blood diseases, we have attempted to find a potential way of using autologous BMT combined with allogeneic BMT. Several problems should be understood before it is used in a clinical setting. First, it should be safe, the recipient immunotolerance to HLA-mismatched hematologic stem cell engraftment could be induced enough to avoid severe GVHD after mixed BMT. Second, it is important to know which donor should be selected, how many allogeneic stem cells should be mixed, and what is the optimal time for allogeneic marrow cell transplantation. Finally, it should increase the long-term disease-free survival in patients with malignant diseases, ie more effective GVL should be induced by MBMT in some way, such as using cytokines. Based on the recent results of immunotolerance studies in animals,<sup>13,14</sup> some investigators have described a murine model for the development of donor-specific tolerance across complete MHC barriers using lethal irradiation followed by reconstitution with a mixture of T cell-depleted (TCD) syngeneic and TCD allogeneic bone marrow.<sup>15</sup> Such animals develop stable mixed chimerism and immunocompetence without clinical evidence of GVHD. Recently, we have studied the graft-versus-leukemia effect of syngeneic marrow mixed with H-2 haploidentical marrow grafting and its tolerance without graft-versus-host disease.<sup>16</sup> By use of spleen enlargement of neonatal mice and mortality of sublethally irradiated adult mice after the i.p. injection of mixed spleen cells, the results showed that syngeneic cells mixed with 1/6 H-2 haploidentical spleen cells significantly reduced GVHD in neonatal mice, and prevented lethal GVHD in sublethally irradiated adult mice. Lethally irradiated 615 (H-2k) mice were given a lethal dose of 615 leukemic cells along with syngeneic marrow or a mixture of syngeneic plus 1/6 H-2 haploidentical marrow cells and the anti-leukemic effects of mixed marrow transplantation were demonstrated. This observation may suggest a potential way to reduce relapse and increase the safety of clinical autologous marrow mixed with HLA-haploidentical marrow transplantation in the treatment of patients with malignant blood disorders.

In this report, 16 cases, including 12 with acute leukemia and four with advanced malignant lymphoma, were treated by autologous marrow, which was purged *in vitro* by hyperthermia following incubation for 5 days with IL-2 in liquid culture<sup>8,17</sup> and mixed with HLA-haploidentical marrow cells from their sibling or parent. Acute GVHD was not observed in any patients, but, cGVHD was observed in eight patients who received the allogeneic marrow cells 2 h after ABMT. The major manifestations of cGVHD were skin lesions, diffuse hyperpigmentation, buccal mucositis, weight loss, chronic liver disorders, failure to thrive, and so on. However, it appeared about 1 month after MBMT and was different from that which occurred following allogeneic BMT, which always appeared 3 months after transplantation.<sup>18</sup> Hematological rescue in the clinical setting was demonstrated in all cases but one, who died early from

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hepatic VOD. Mixed chimerism was found in three cases, by analysis of sex chromosomes. Comparison of survival for patients who received allogeneic BMT 6 h after ABMT with those 2 h after ABMT revealed that in the former group, five of eight patients relapsed with a median of 4 months, and died within 5 to 14 months. Two died from HVOD and encephalopathy at 3 weeks and 7 months, respectively and only one patient is still DFS for more than 40 months. However, in the latter group, none relapsed with median follow-up of 12 months, all patients but two, who died from fulminant hepatitis and appendicular perforation at 3 or 8 months after MBMT, respectively, are still alive in DFS. Fifteen patients were successfully engrafted after MBMT. One patient died from HVOD before complete hematologic reconstruction could occur. One patient had persistent pancytopenia for 8 months after increasing leukocytes and platelets in the short period following MBMT for cGVHD.

Beaujean *et al*<sup>8</sup> have shown that lymphokine-activated killer cells generated by IL-2 treatment of peripheral blood and marrow are able to lyse leukemic blasts, and have been successfully used in clinical ABMT for treatment of patients with acute lymphoblastic leukemia.<sup>17</sup> Sykes *et al*<sup>10,11</sup> have demonstrated that a short course of IL-2 beginning at the time of allo-BMT can reduce both acute or chronic GVHD mortality in mice without preventing allo-engraftment or attenuating the GVL effects of allogeneic T cells. The mechanisms might be due to an inhibitory effect on the GVHD promoting activity of donor CD4<sup>+</sup> cells, without reducing graft-versus-leukemia effects of CD4<sup>+</sup> or CD8<sup>+</sup>.<sup>12</sup> Studies also show in the experimental and clinical settings that GVHD and GVL effects of T cells can be dissociated.<sup>12</sup> Several mechanisms have been proposed to explain this dissociation, including the recognition of tumor-associated or tissue-associated antigens, and so on.<sup>12,19</sup> Recently, Sykes *et al*<sup>12</sup> have demonstrated that the different CD4<sup>+</sup> activities promote GVHD and GVL effects. The former, but not the latter, activities are selectively inhibited by treatment with IL-2, and the IL-2-induced GVHD protection is independent of host T cell reactivity against the donor. In our studies, all the recipients received IL-2 treatment for 2 weeks following MBMT. All patients but one, who died from HVOD early, were successfully engrafted after MBMT. Mixed chimerism was found in three cases, who had different sex donors, by analysis of sex chromosomes for more than 9 months. However, acute GVHD was not observed in any patients (group 1 and 2) after MBMT, but chronic GVHD (cGVHD) was seen in all patients in group 2, who had not relapsed at the time of this report; the latter is considered to be related to GVL effects. Because the follow-up time was short, study on the effects of GVL in this respect are still now in progress in our clinic. These observations suggest that treatment with IL-2 in MBMT recipients might provide several possible advantages in MBMT, including the marked diminution of GVHD, and probably augmentation of GVL effects.

In conclusion, the clinical observations of using autologous marrow, which was purged *in vitro* by hyperthermia (42.5°C for 70 min) following incubation for 5 days with IL-2 in liquid culture, mixed with HLA-haploidentical allogeneic marrow transplantation for treatment of 16 patients

with malignant blood diseases are reported. The results show that this mixed transplantation is, especially in recipients who received HLA-haploidial marrow 2 h after ABMT, associated with a lower incidence of acute GVHD, fewer complications, engraftment with partial mixed chimerism and a low relapse rate. Therefore, it is a safe, effective and new approach to treat patients with malignancies. In order to detect the effects of GVL, prospective studies will be performed to confirm these findings.

### Acknowledgements

We wish to thank all the doctors and nurses in the department of Hematology and Bone Marrow Transplantation Center of Lanzhou General Hospital for participating in this work.

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**EXHIBIT C****United States Patent [19]**

Slavin



US006143292A

[11] Patent Number: 6,143,292

[45] Date of Patent: Nov. 7, 2000

[54] ALLOGENEIC CELL THERAPY FOR  
CANCER FOLLOWING ALLOGENEIC STEM  
CELL TRANSPLANTATION

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[22] PCT Filed: May 24, 1996

[86] PCT No.: PCT/US96/07652

§ 371 Date: Nov. 21, 1997

§ 102(e) Date: Nov. 21, 1997

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PCT Pub. Date: Nov. 28, 1996

**Related U.S. Application Data**

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25, 1995, abandoned.

[51] Int. Cl. .... A61K 35/28; C12N 5/08

[52] U.S. Cl. .... 424/93.7; 424/93.71; 424/85.5;  
424/85.7; 424/85.2; 424/85.4; 424/144.1;  
424/577; 424/578; 435/325; 435/375

[58] Field of Search .... 424/93.71, 93.7,  
424/85.5, 85.7, 85.2, 85.4, 144.1, 577,  
578; 435/325, 372

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Primary Examiner—Phuong T. Bui  
Attorney, Agent, or Firm—Townsend and Townsend and  
Crew LLP

**[57] ABSTRACT**

A method of treating a human cancer patient having a solid tumor comprising malignant cells is disclosed, wherein the patient having undergone a cancer therapy regimen comprising allogeneic stem cell transplantation. The method comprises administering allogeneic lymphocytes to the patient and monitoring the patient for levels of malignant cells.

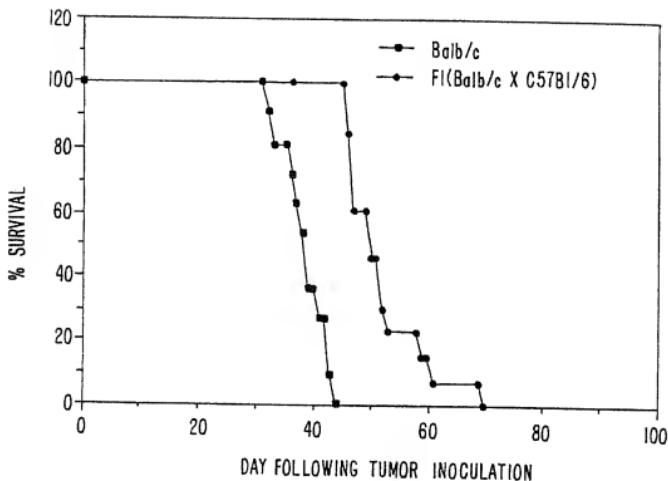


FIG. 1.

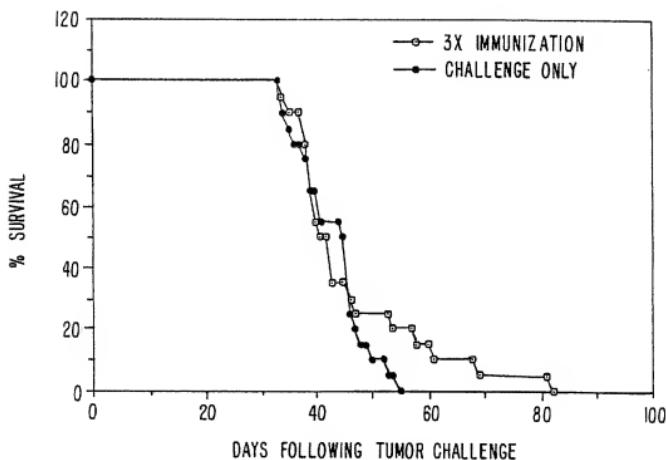


FIG. 2.

EXHIBIT C

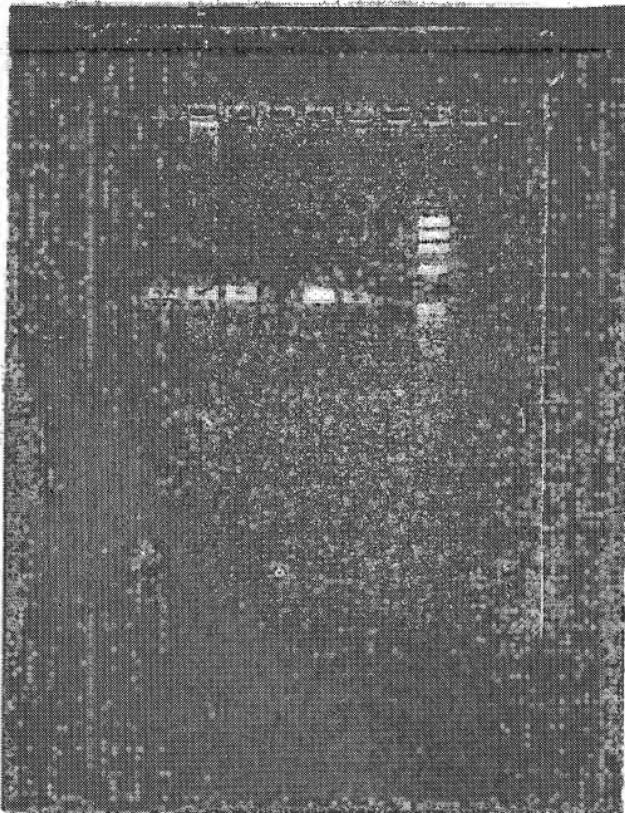
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1 2 3 4 5 6 7 8



*FIG. 3.*

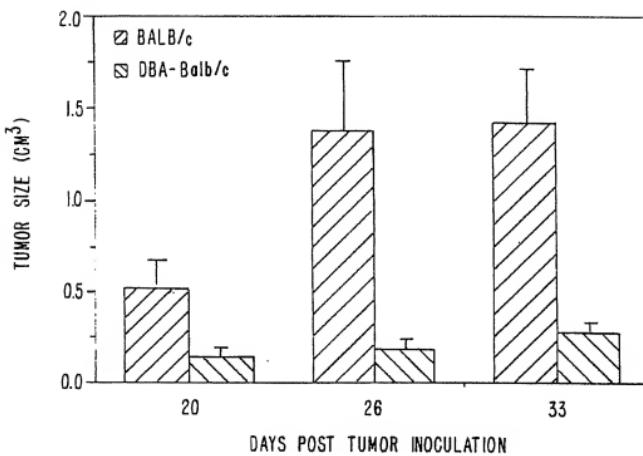


FIG. 4.

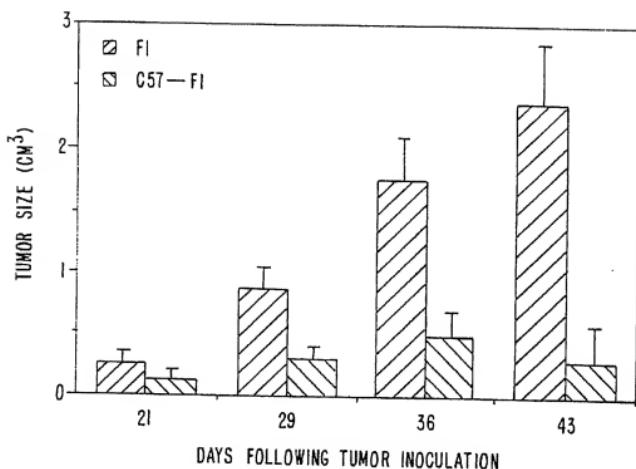


FIG. 5.

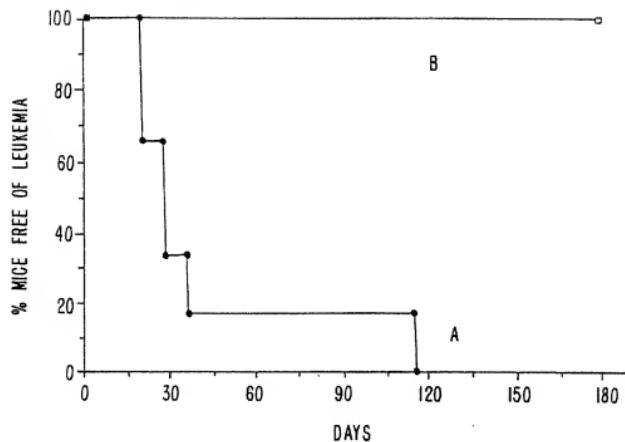


FIG. 6.

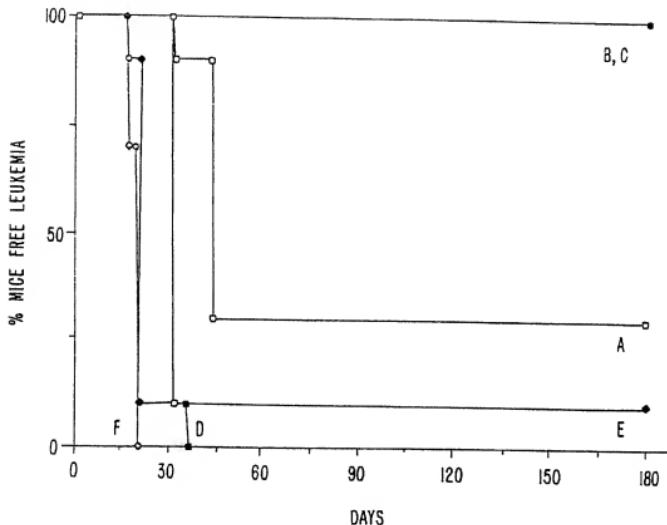


FIG. 7.

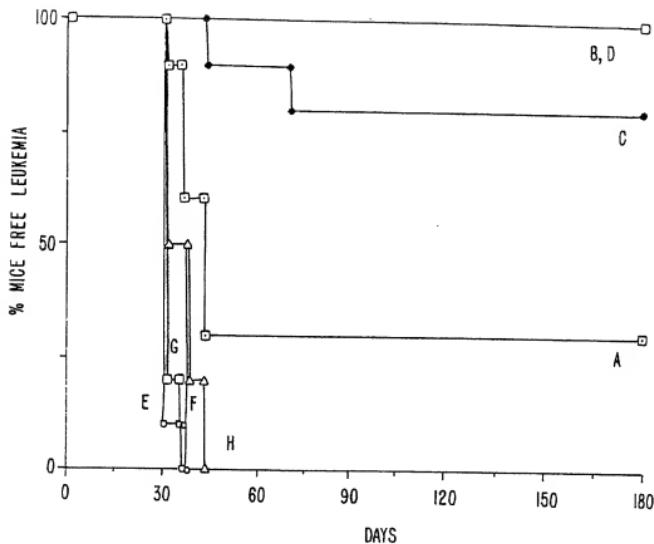


FIG. 8.

**ALLOGENEIC CELL THERAPY FOR  
CANCER FOLLOWING ALLOGENEIC STEM  
CELL TRANSPLANTATION**

This is a continuation in part of U.S. application Ser. No. 08/449,764, filed May 25, 1995, now abandoned.

**FIELD OF THE INVENTION**

This invention relates to a methods for eradicating tumor cells that remain viable in a patient following allogeneic stem cell transplantation. More particularly, this invention relates to use of allogeneic lymphocytes for eradication of solid tumor cells following allogeneic stem cell transplantation. The invention also relates specifically to use of allogeneic activated donor lymphocytes for treatment of cancer patients, including relapsing patients.

**BACKGROUND OF THE INVENTION**

Patients suffering from malignant hematological disorders such as leukemia or lymphoma may, under appropriate circumstances, be administered autologous or allogeneic bone marrow transplants as part of a therapeutic regimen. Such transplants also can be useful in conjunction with therapy of non-hematological malignancies such as breast carcinomas or other solid tumors. Bone marrow transplantation makes it possible to administer to patients with resistant disease high, "supra-lethal" combinations of chemotherapy and/or radiation, ignoring the irreversible toxicity of such therapeutic combinations on the normal bone marrow compartment. Nevertheless, such "debulking" of a patient's tumor can leave a fraction of residual malignant cells that may lead to disease relapse.

Several lines of evidence suggest that a significant portion of the beneficial effect of allogeneic bone marrow transplantation (i.e., bone marrow transplantation from an individual not genetically identical to the host patient) stems from cell-mediated interactions of immune cells of donor origin against residual tumor cells in the host that have escaped the chemotherapy/radiation debulking regimen. Following allogeneic bone marrow transplantation (Allo-BMT), the incidence of relapse is significantly lower in leukemia patients with clinical manifestations of acute or chronic graft versus host disease (GVHD), as compared with patients with no GVHD, indicating that immune-mediated allogeneic interactions of immunocompetent cells of donor origin against the host can be accompanied by graft vs. leukemia (GVL) effects.

Higher relapse rates seem to occur in patients undergoing Allo-BMT with T-lymphocyte depletion for prevention of GVHD, compared to recipients of non-T-cell depleted marrow allografts, regardless of the severity of GVHD. Likewise, relapse rates in patients with acute leukemia or chronic myelogenous leukemia reconstituted by bone marrow grafts obtained from an identical twin (syngeneic grafts) are significantly higher than in those reconstituted by bone marrow cells obtained from an HLA-identical but non-syngeneic sibling. Similarly, relapse rates following transplantation of the patient's own (autologous) marrow, even following adequate purging *in vitro* for elimination of residual leukemia cells, are significantly higher than following Allo-BMT.

Recent studies by several groups have demonstrated that chronic myelogenous leukemia (CML) patients who relapse following Allo-BMT can be treated successfully by infusion of resting (i.e., unactivated by *in vitro* treatment with T-cell activators such as cytokines) HLA-matched leukocytes from

the Allo-BMT donor in order to achieve a second remission. Slavin et al., Blood 72 (Suppl. 1): 407a (1988); Kohl et al., Blood 76:2462 (1990); Baer et al., J. Clin. Oncology 11:513 (1993); Jiang et al., Bone Marrow Transpl. 11:133 (1993); Drohyski et al., Blood 82:2310 (1993); Antin, Blood 82:2273 (1993); Porter et al., N. Engl. J. Med. 330:100-06 (1994).

The therapeutic effects of the infused leukocytes are mediated by potentiation of GVL effects, induced following 10 Allo-BMT, by immunocompetent donor T-cells that are not tolerant to the malignant hematopoietic cells. Slavin et al., Blood 72 (Suppl. 1): 407a (1988); Slavin et al., Bone Marrow Transpl. 6:155-61 (1990); Kohl et al., Blood 76:2462 (1990); Baer et al., J. Clin. Oncology 11:513 (1993); Jiang et al., Bone Marrow Transpl. 11:133 (1993); Drohyski et al., Blood 82:2310 (1993); Antin, Blood 82:2273 (1993); Porter et al., N. Engl. J. Med. 330:100-06 (1994). Unfortunately, only about 50-70% of the CML patients relapsing post Allo-BMT respond favorably to 20 Allo-CT. Kohl et al., Clin. Blood 82 (Suppl. 1):840 (1993). Moreover, long-term disease free survival is far from optimal due to response failures, subsequent relapses and complications arising from GVHD and marrow aplasia.

Finally, the possible anti-solid tumor effects of allogeneic 25 lymphocytes following Allo-BMT have been relatively unknown compared to the documented effects of allogeneic lymphocytes on malignant hematopoietic cells.

**SUMMARY OF THE INVENTION**

30 The present invention includes a method of treating a human cancer patient who has undergone a cancer therapy regimen including allogeneic stem cell transplantation. The term "stem cell transplantation" as used herein includes infusion into a patient of hematopoietic stem cells derived from any appropriate source of stem cells in the body. The stem cells may be derived, for example, from bone marrow, from the peripheral circulation following mobilization from the bone marrow, or from fetal sources such as fetal tissue, fetal circulation and umbilical cord blood. "Bone marrow transplantation" is considered herein to be simply one form of stem cell transplantation. Mobilization of stem cells from the bone marrow can be accomplished, for example, by treatment of the donor with granulocyte colony stimulating factor (G-CSF) or other appropriate factors (e.g., IL-8) that induce movement of stem cells from the bone marrow into the peripheral circulation. Following mobilization, the stem cells can be collected from peripheral blood by any appropriate cell pheresis technique, for example through use of a commercially available blood collection device as exemplified by the CS 3000® Plus blood cell collection device marketed by Baxter Healthcare Corporation. Methods for performing apheresis with the CS 3000® Plus machine are described in Williams et al., Bone Marrow Transplantation 5: 129-33 (1990) and Hillery et al., Transfusion 33: 316-21 (1993), both publications being incorporated herein by reference.

Infusion of the hematopoietic stem cells may result in complete and permanent engraftment (i.e., 100% donor hematopoietic cells), or may result in partial and transient engraftment, provided the donor cells persist sufficiently long to permit performance of allogeneic cell therapy as described herein. Thus, the term "stem cell transplantation" covers stem cell infusion into a patient resulting in either 40 complete or partial engraftment as described above.

As used herein, the term "Allo-CT" (allogeneic cell 45 therapy) refers to infusion of resting allogeneic

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lymphocytes, i.e., lymphocytes that have not been previously exposed to T-cell activator in vitro; the term "Allo-ACT" (allogeneic activated cell therapy) refers to infusion of allogeneic lymphocytes preactivated in vitro with a T-cell activator such as recombinant human interleukin-2 (rhIL-2). Such activated donor lymphocytes are herein termed "ADL." It is to be understood that the allogeneic lymphocyte infused in a patient need not be infused as a purified T-cell preparation. Although it is possible to infuse a relatively pure T-cell preparation, the cells may be infused in the form of a peripheral blood mononuclear cell (PBMC) preparation. For example, the PBMC preparation obtained as a result of phresis with the CS 3000® Plus blood cell collection device is appropriate for the present invention. Such a cell preparation is approximately 95% mononuclear cells, the majority of which are T cells. In appropriate circumstances it is even possible to administer allogeneic lymphocytes to the patient by simply providing whole blood.

Both Allo-CT and Allo-ACT may be performed with or without accompanying in vivo administration of a T-cell activator. Typically the infused allogeneic lymphocytes are derived from the same donor who provided the stem cells for allogeneic stem cell transplantation. However, the infused lymphocytes may be derived from other donors in appropriate circumstances. For example, if the infused lymphocytes are lifespan-limited as described below, the same or a different donor can provide the cells, depending on the clinical setting.

The term "cancer" as used herein includes all pathological conditions involving malignant cells; this can include "solid" tumors arising in solid tissues or organs (i.e., tumor cells growing as multi-cellular masses supported by blood vessels), as well as tumor cells originating from hematopoietic stem cells.

The invention features a method of treating human cancer patients with solid tumors, including without limitation breast carcinomas, composed of malignant cells. The patients have undergone allogeneic stem cell transplantation. Post-transplantation, the patients are infused with allogeneic lymphocytes in order to induce a graft-versus-tumor response in the patient. The infused allogeneic lymphocytes can be activated, prior to infusion, by in vitro exposure to a T-cell activator. Whether or not the lymphocytes are activated prior to infusion, the patient also can be provided with T-cell activator in vivo in order to provide continuing activation stimulus to the lymphocytes after infusion.

The T-cell activator comprises at least one T-cell signal transduction pathway activator. The T-cell activator may include, without limitation, one or more of the following signal transduction pathway activators: interleukin-1 (IL-1), interleukin-2 (IL-2) interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-12 (IL-12), interleukin-13 (IL-13), interleukin-15 (IL-15), interferon-alpha (IFN $\alpha$ ), interferon-gamma (IFN $\gamma$ ), tumor necrosis factors such as TNF $\alpha$ , anti-CD3 antibodies (anti-CD3), anti-CD28 antibodies (anti-CD28), phytohemagglutinin, concanavalin-A and phorbol esters. The T-cell activators can be native factors obtained from natural sources, factors produced by recombinant DNA technology, chemically synthesized polypeptides or other molecules, or any derivative having the functional activity of the native factor. Most preferably the T-cell activator is IL-2, for example recombinant human IL2 (rhIL-2). The T-cell activator used to activate the donor lymphocytes in vitro, and the T-cell activator used for in vivo administration, may be the same or different T-cell signal transduction pathway activators.

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The allogeneic lymphocytes may be provided to the patient in a series of incrementally increasing amounts, with the patient monitored for signs of GVHD between increments. If no GVHD manifests, or if the GVHD is not severe and is controllable with standard anti-GVHD prophylaxis, then the patient can be administered an incrementally larger dose of allogeneic lymphocytes than was provided in the previous infusion. Typically, though not necessarily, the dosages are adjusted by log increments, e.g., 10<sup>5</sup>, 10<sup>6</sup>, 10<sup>7</sup> lymphocytes/kg and so on. Preferably the allogeneic lymphocytes are HLA-compatible (see below) with the patient, although this is not necessary in all cases, particularly if the infused lymphocytes are lifespan-limited. For example, the allogeneic lymphocytes may carry a "suicide gene," allowing the cells to be killed after infusion into the patient, through use of a chemotherapeutic agent. After infusion of the allogeneic lymphocytes, the patient is monitored for levels of malignant cells.

The invention also includes treatment of human cancer patients having malignant hematopoietic cells, for example patients with chronic myelogenous leukemia or acute lymphocytic leukemia. As in the case with solid tumor patients, these patients have undergone an allogeneic stem cell transplantation procedure as part of a regimen to treat the malignancy. Following allogeneic stem cell transplantation, the patient is infused with allogeneic lymphocytes that have been activated in vitro by exposure to a T-cell activator prior to administration to the patient. Following infusion, the patient is monitored for levels of malignant hematopoietic cells. The patient also can be provided with in vivo T-cell activator. The T-cell activator, whether used for in vitro activation or administered in vivo, can be as described above for the solid tumor embodiments.

The present invention is particularly useful for those unfortunate patients who, in spite of an allogeneic stem cell transplant, continue to exhibit malignant cells as evidenced by overt relapse or other indication that malignant cells have not been completely eradicated. The methods are further applicable to patients who have not only failed to respond to an allogeneic stem cell transplant, but who have also failed to respond to a post-transplant cell therapy regimen including infusion of allogeneic resting donor lymphocytes (Allo-CT).

In one embodiment, the patient is administered about 10<sup>5</sup> cells/kg to about 10<sup>9</sup> cells/kg of allogeneic ADL and is then monitored for levels of malignant cells. In an alternative embodiment, the patient also can be administered T-cell activator in vivo, for example by injection in concert with a pharmaceutically acceptable carrier. Preferably the T-cell activator is given to the patient over a time course of two to seven days, more preferably two to five days, and most preferably two to four days. The T-cell activator may be administered beginning on the same day as infusion of the allogeneic activated donor lymphocytes.

Preferably the allogeneic donor lymphocytes are HLA-compatible with the patient. HLA-compatible lymphocytes include cells that are fully HLA-matched with the patient. Alternatively the HLA-compatible cells should be at least haploididentical with the patient. If the HLA-compatible lymphocytes are derived from a sibling of the patient, the cells preferably are fully HLA-matched with the patient, although some mismatch may be tolerated. For example, the HLA-compatible lymphocytes from a sibling may, in some cases, be single HLA locus-mismatched. If the HLA-compatible lymphocytes are derived from an unrelated individual, preferably the cells are fully HLA-matched with the patient.

The present invention also includes the use of allogeneic donor lymphocytes, unactivated or in vitro-activated, as well

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as T-cell activators, in the manufacture of a medicament for the treatment of human cancer patients as described above. The invention further includes an article of manufacture comprising packaging material and a container within the packaging material. The packaging material contains a label or package insert indicating that the contents of the container may be used for the treatment of human cancer patients as described above. The container may be a collapsible container comprising opposing walls of flexible material and a flexible tube protruding from the container. The contents of the container may include unactivated lymphocytes or ADL that are allogeneic with respect to the patient to be treated. Alternatively, the container may include a T-cell activator.

### BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Percent survival as a function of days following intradermal inoculation of 4T1 tumor cells ( $10^6$ ) into 13 BALB/c or F1 mice in 3 separate experiments per each strain of mice.

FIG. 2. Percent survival as a function of days following an intradermal challenge dose of ( $10^6$ ) 4T1 cells given to naive BALB/c mice ( $n=20$ ) or to BALB/c mice ( $n=20$ ) pre-immunized intradermally 3 times with  $10^7$  irradiated 4T1 cells given in intervals of 7–10 days. The challenge dose was injected 7 days after the 3rd immunizing dose.

FIG. 3. Detection of y-chromosome by PCR analysis. Peripheral blood samples were taken from female recipient BALB/c mice 1,3,6 and 9 months following hematopoietic reconstitution with male DBA/2 donor cells (Lanes 1–4, respectively). Lane 5: male positive control direct PCR. Lane 6: positive DNA control. Lane 7: no DNA control. Lane 8: Hae III size markers. The intensity of the signal depends on the number of cells, which was different in each sample.

FIG. 4. 4T1 tumor size as function of days following tumor inoculation into 13 naive BALB/c mice and 15 chimeric BALB/c mice reconstituted with DBA/2 hematopoietic cells (DBA—BALB/c). Tumor cells were inoculated into chimeric mice 60–90 days following bone marrow cell reconstitution. Results represent 3 separate experiments.

FIG. 5. 4T1 tumor size as function of days following tumor cell inoculation into 18 naive F1 (BALB/c $\times$ C57Bl/6) mice and 11 chimeric F1 mice reconstituted with C57Bl/6 hematopoietic cells (C57—F1). Tumor cells were inoculated into chimeric mice 60–90 days following bone marrow cell reconstitution. Results represent 3 separate experiments.

FIG. 6. Development of leukemia in normal BALB/c control mice (group A,  $n=10$ ) and in B6 $\rightarrow$ BALB/c chimeric mice (group B,  $n=12$ ) inoculated intravenously with  $10^6$  BCL1 cells.

FIG. 7. Time intervals needed for effective GVL effects: development of leukemia in secondary adoptive recipient BALB/c mice after receiving  $10^6$  spleen cells obtained from B6 $\rightarrow$ BALB/c chimeras inoculated with  $10^6$  BCL1 cells at 7 days (group A), 14 days (group B) and 21 days (group C) prior to adoptive transfer; and from normal BALB/c mice inoculated with  $10^6$  BCL1 cells: 7 days (group D), 14 days (group E) and 21 days (group F) prior to adoptive transfer. Each group consisted of 10 mice.

FIG. 8. Amplification of GVL effects by allogeneic spleen cells and rHL-2: development of leukemia in secondary adoptive recipient BALB/c mice after receiving  $10^6$  spleen cells obtained from B6 $\rightarrow$ BALB/c chimeras or normal BALB/c mice 7 days post-inoculation with  $10^6$  BCL1 cells. Group A: untreated chimeras; group B: chimeras injected with rHL-2; group C: chimeras infused with spleen cells;

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group D: chimeras infused with spleen cells and with rHL-2; group E: normal BALB/c controls without further treatment; group F: normal BALB/c controls injected with rHL-2; group G: normal BALB/c controls infused with spleen cells; group H: normal BALB/c controls infused with spleen cells and with rHL-2. Each experimental group consisted of 10 mice.

### DETAILED DESCRIPTION OF THE INVENTION

A series of animal experiments was undertaken in order to evaluate 1) the ability of allogeneic lymphocytes to effect a solid tumor response following allogenetic stem cell transplantation, and 2) the feasibility and efficacy of Allo-ACT with or without T-cell activator administration *in vivo*, following allogeneic stem cell transplantation. The concepts developed in the animal experiments were also extended into the clinical setting to demonstrate efficacy in human breast cancer patients as well as with human cancer patients non-responsive to Allo-BMT and Allo-CT.

The animal experiments discussed below demonstrate the feasibility of inducing an immune-mediated graft-versus tumor (GVT) effect in solid tumors, using a murine model of mammary adenocarcinoma derived from BALB/c(H-2<sup>b</sup>) mice. A murine breast cancer cell line (4T1) was used that is highly tumorigenic in syngeneic (BALB/c) or haplodentical F1 (BALB/c $\times$ C57Bl/6) (F1) mice, is only partially tumorigenic in an H-2<sup>c</sup> congenic strain of mice (DBA/2) and is non-tumorigenic in an unrelated MHC (H-2<sup>d</sup>) strain of mice (C57Bl/6). 4T1 cells express on their surfaces class I major histocompatibility (MHC) antigens, adhesion molecules and CD44 homing-associated adhesion molecules, but do not express MHC class II antigens or costimulatory molecules such as B7.

Female BALB/c (H-2<sup>b</sup>) or F1 (H-2<sup>b/b</sup>) mice were reconstituted with male minor mismatched DBA (H-2<sup>a</sup>-derived bone marrow cells or with major mismatched C57 (H-2<sup>d</sup>)-derived bone marrow cells, respectively, 24 hr following lethal total body irradiation. Recipient mice carrying minor or major mismatched grafted donor cells were inoculated with 4T1 tumor cells 2–3 months following bone marrow reconstitution. The allogeneic donor cells, whether differing from the tumor cells in minor or MHC antigens, were able to affect development of the primary tumor, which expressed host-type MHC alloantigens. Tumor size in bone marrow chimeras across minor or MHC antigens was significantly ( $p<0.05$ ) smaller than tumor size observed in BALB/c or F1 ungrafted control mice. These results demonstrate that it is possible to induce a GVT effect by alloreactive cells in a murine model of mammary carcinoma.

Previous studies (Cohen et al., *J. Immunol.* 151: 1803–10 (1993), incorporated herein by reference) have demonstrated that use of *in vitro* activated donor lymphocytes (ADL) with or without *in vivo* rHL-2 (Allo-ACT $\times$ rHL-2) provides significant GVL effects in mice. However, since 100% survival was observed in all the mice that were administered allogeneic lymphocytes, it was not possible to identify particular enhancements of GVL effects through use of ADL or *in vivo* rHL-2. This study also provided confirmation that the GVL effects are caused predominantly by allogeneic T cells and not by natural killer (NK) cells which were considered until recently as being MHC non-restricted. Alloreactive NK cells, B cells and macrophage cells may, however, play a role in the GVL or GVT effects induced by allogeneic T cells. The results further indicated that the GVL effects are not due to the cascade of allogeneic responses,

inflammatory reactions and *in vivo* cytokine release that results from GVHD per se.

In a further set of experiments reported below, BALB/c, C57BL/6 (B6) and (BALB/cxBG)F1(F<sub>1</sub>) mice were used to evaluate various allogeneic cell therapy protocols accompanied or unaccompanied by *in vivo* administration of a T-cell activator. BCL1 cells, representing a spontaneous B-cell leukemia/lymphoma of BALB/c origin originally described by Slavin and Strober, *Nature* 272:624 (1978), were used as a tumor model. Infusion of 10 to 100 BCL1 cells in BALB/c mice results in a typical cell leukemia/lymphoma characterized by splenomegaly with subsequent peripheral blood lymphocytosis and death in 100% of recipients. BCL1 causes leukemia in F<sub>1</sub> recipients also, but takes longer to develop as compared with BALB/c recipients. The present inventor investigated the susceptibility of well-established and fully reconstituted tolerant B6→BALB/c chimeras to BCL1 cells. Chimeras were generated by lethally irradiating BALB/c mice and reconstituting 24 hours later with T-cell depleted B6 bone marrow cells. None of the chimeras showed any clinical evidence of GVHD. Normal BALB/c mice and B6→BALB/c chimeras were injected intravenously with 10<sup>4</sup>, 10<sup>5</sup>, or 10<sup>6</sup> BCL1 cells. All normal BALB/c mice developed leukemia within 21–58 days and died, whereas all well-established chimeras (i.e., remaining chimeric 2–3 months after induction of chimerism) survived with no evidence of disease for more than 6 months. In contrast, previous studies showed that early inoculation of BCL1 into B6→BALB/c or B6→F1 recipients resulted in leukemia in all recipients with MRD. Weiss et al., *Cancer Immunol. Immunother.* 31: 236 (1990).

Adoptive transfer experiments were performed with both the normal BALB/c and the B6→BALB/c chimeras that had been injected with 10<sup>6</sup> BCL1 cells. Spleen cells (10<sup>6</sup>) were transferred to 10 secondary naive BALB/c mice at 7, 14 and 21 days post-inoculation of the BCL1 cells. Seven out of 10 secondary recipients receiving cells from chimeras removed 7 days after inoculation with BCL1 developed leukemia within 44 days. In contrast, none of the secondary recipients receiving cells obtained from chimeras inoculated with BCL1 cells 14 and 21 days prior to cell transfer developed leukemia. The data suggest that a period of at least 14 days is required for complete eradication of 10<sup>6</sup> BCL1 cells, whereas at 7 days eradication of leukemic cells is still incomplete.

Further experiments were conducted in the chimeras to determine the effects of *in vivo* administration of T-cell activator (rhIL-2). Chimeras were injected with 10<sup>6</sup> BCL1 cells and then variously treated with *in vivo* rhIL-2, lymphocytes or combinations of rhIL-2 and lymphocytes. After seven days, all the mice were sacrificed and spleen cells were used for adoptive transfer into secondary BALB/c recipients, as above.

All of the secondary BALB/c recipients who received spleen cells from the control normal BALB/c mice developed leukemia. Furthermore, no antileukemic effects were detected in normal control BALB/c mice treated with rhIL-2, allogeneic splenocytes or both. In contrast, 70% of the chimeras in the group without any additional treatment did not develop leukemia for a period greater than 6 months. Of the 30% that developed leukemia, the onset was delayed to 44–52 days. Of the chimeras that received only B6 lymphocytes, 80% remained disease free and the remaining 20% showed delayed onset of leukemia. Of the chimeras that were treated with rhIL-2, or that were treated with both rhIL-2 and B6 lymphocytes, 100% were disease free for more than 6 months.

Taken together, these results indicate that chimeras generated by irradiating BALB/c mice and reconstituting with T-cell depleted B6 bone marrow cells are capable of resisting the leukemogenic potential of BCL1 cells (which are of BALB/c origin), assuming chimerism is established and the recipients are immunocompetent. This is in spite of the fact that the chimeras are fully tolerant to BALB/c alloantigens, since such chimeras have been shown to be fully tolerant to host (BALB/c) alloantigens and to accept donor-type skin allografts indefinitely. Levine and Reisner, *Transplantation* 55:3 (1993). Moreover, the chimeras are resistant to the BCL1 cells in the absence of GVHD. Thus, the antitumor effects in tolerant chimeras can include recognition of tumor-associated or tumor-specific cell surface determinants other than host-type major histocompatibility complex (MHC) determinants, independently of GVHD. Significantly, enhancement of GVL effects can be achieved, without GVHD or, alternatively, with controllable GVHD, by post-transplant administration of ADL or without a short course of relatively low-dose rhIL-2.

It is especially advantageous to use graded increments of allogeneic cells while controlling for GVHD. The greater the time interval from BMT to cell therapy, the less likely is the development of uncontrollable GVHD and the larger the number of allogeneic donor T cells that can be given. See Slavin et al., *J. Exp. Med.* 147: 963 (1978); Slavin et al., *Cancer Invest.* 10: 221–7 (1992). This may be contrasted to mice with residual tumor cells given allogeneic T cells during the early post-BMT period. The infused allogeneic T cells in these cases do not become tolerant to the host, resulting in GVHD. Thus, infusion of allogeneic lymphocytes, especially following *in vitro* and *in vivo* activation of donor T cells by T-cell activators such as rhIL-2, makes it possible to infuse, relatively late post-BMT, non-tumor-tolerant donor T cells that are accepted by the recipient but that engender potent GVL effects. The T cells may be given in graded increments, with proportionately more cells administered as the time from BMT increases.

The results and indications deriving from the mouse experiments were extended into the clinical setting with human patients suffering from breast cancer and from malignant hematological disorders, including acute and chronic leukemias. Specifically, the present inventor has discovered that a therapeutic regimen of Allo-CT can be effective in treating breast cancer following allogeneic BMT. The present inventor has also discovered that activated donor lymphocytes can provide anti-tumor effects even beyond those obtainable with unactivated allogeneic lymphocytes. For example, Allo-ACT and *in vivo* T-cell activator can be used successfully in a clinical setting to treat relapse following Allo-BMT. Thus, results in human patients provide important confirmation and extension of the animal data reported above.

More particularly, the present inventor has discovered that *in vitro* activation of donor's PBL prior to infusion into the patient provides a means to induce remission following an unsuccessful regimen of bone marrow transplantation and cellular immunotherapy. Surprisingly, donor's PBL activated *in vitro* provided a measurable GVL effect when the same cells, absent such *in vitro* treatment, failed to eradicate the tumor cells. It is noteworthy that, in some cases, these unactivated cells, even though not exposed to T-cell activator prior to infusion, nevertheless were exposed to activating T-cell activator *in vivo* following infusion. In contrast, PBL from the same donor were effective when preactivated prior to infusion (Allo-ACT approach) and accompanied by *in vivo* T-cell activator. In addition, it is demonstrated herein

that an Allo-ACT regimen can be undertaken in this setting without necessarily inducing clinically significant GVHD.

To document the ability of allogeneic lymphocytes to provide a therapeutic effect in solid tumor patients, a human patient with acute myelogenous leukemia (AML) and recurrent breast cancer was treated with induction chemotherapy, allogeneic stem cell transplantation and post-transplant allogeneic cell therapy (see Example 3 below). The approach in treating this patient was oriented towards AML, although most of the components used for induction chemotherapy are known to be active against breast cancer as well. Nonetheless, the dose intensity was less than optimal for treatment of recurrent breast cancer, and it would not be expected that a patient with an aggressive recurrent breast cancer would respond to such "suboptimal" chemotherapy. The breast cancer response therefore can be attributed to this allogeneic cell-mediated immunotherapy received by this patient.

As an illustrative example to demonstrate the clinical efficacy of the Allo-ACT plus *in vivo* T-cell activator treatment regimen, a human patient with chronic myelogenous leukemia (CML) having a very poor prognosis was treated (see Patient No. 1 in Example 3, below).

Chronic myelogenous leukemia (CML) is a hematological disorder that is the result of neoplastic transformation of pluripotent stem cells. The Philadelphia (Ph) chromosome was first described in 1960 as an abbreviated chromosome found in the bone marrow of patients with CML. The Ph chromosome is the result of a reciprocal translocation between the long arms of chromosomes 9 and 22. The potential breakpoints on chromosome 22 occur in a small 5.8 kb region called the breakpoint cluster region (bcr). The breakpoint cluster region is part of a larger bcr gene that contains four exons. Potential breakpoints on chromosome 9 are scattered over a distance of at least 100 kb, but are all located 5' to the c-abl proto-oncogene. The Ph translocation transfers the c-abl gene from its position on chromosome 9 to the Ph chromosome. Because 90% of CML patients carry the Ph chromosome, it constitutes the hallmark of CML and is diagnostic of the disease. Approximately 5% of the childhood and 30% of the adult acute lymphocytic leukemias (ALL) also carry the Ph chromosome. The Ph chromosome in CML and ALL results from the same translocation of c-abl to different introns of the bcr gene.

Elimination of cells displaying the Ph karyotype is one indication of remission. An alternate method of assaying for presence of the Ph chromosome is through use of the polymerase chain reaction (PCR) to detect the bcr/abl transcript. Elimination of the bcr/abl transcript upon PCR analysis is indicative of successful elimination of cells leading to CML.

Prior to treatment, the patient (Patient No. 1 in Example 3, below) had relapsed following Allo-BMT and had remained positive for CML markers following a course of Allo-CT accompanied by *in vivo* treatment with rhIL-2. The patient had not experienced GVHD as a result of the bone marrow transplant or of the Allo-CT/rhIL-2 regimen. Peripheral blood leukocytes (PBL), taken from the same HLA-matched brother who donated cells for the Allo-BMT, were preactivated *in vitro* by incubation with rhIL-2. The activated donor lymphocytes were administered at a dose between  $10^7$  and  $10^8$  cells per kilogram body weight. This was followed immediately by a three-day course of rhIL-2 administered *in vivo* in order to provide a further stimulus to activation following infusion of cells to the patient.

The patient so treated has not experienced any clinical laboratory signs of GVHD, and has a completely normal

bone marrow morphology. Significantly, following the Allo-CT/rhIL-2 regimen, the PCR test for the presence of the bcr/abl fusion product became negative. As of 28 months post-treatment, the patient displays no evidence for the Ph chromosome, either by cytogenetic or PCR analysis. Additional patients given allogeneic cell therapy are provided in Example 3, below.

In a preferred embodiment for treating human patients with solid tumors, the donor's PBL, unactivated or activated *in vitro*, are infused into the patient following allogeneic stem cell transplantation. Generally the donor's PBL are infused after the patient has attained at least partial hematopoietic recovery from the stem cell transplant; in many cases, the greater the time interval from stem cell transplantation to administration of donor's PBL, the more lymphocytes can be provided since the risk of uncontrollable GVHD is proportionately less at later times post-transplant. The patient may be administered graded increments of donor's PBL, typically beginning with  $10^5$  or  $10^6$  T cells/kg and progressing at log increments, e.g.,  $10^7$ ,  $10^8$ ,  $10^9$  T cells/kg pending no or minimal (controllable) GVHD following the previous infusion. If used, proportionately fewer activated donor lymphocytes are administered compared to the corresponding unactivated donor's PBL. This is because activated lymphocytes, though possibly engendering a heightened anti-tumor effect compared to unactivated lymphocytes, may also put the patient at a somewhat higher risk of GVHD. It is to be noted, however, that if ADL with a limited lifespan are used, then the risk of GVHD is mitigated and larger numbers of ADL may be used. For example, as discussed below, allogeneic lymphocytes may be transduced with a "suicide" gene construct that allows the infused cells to be selectively killed after they have exerted the anti-tumor cell effect in the patient.

For activation of donor's PBL, the cells are incubated in rhIL-2 at a concentration of 60 IU/ml to 12,000 IU/ml, preferably at 600 IU/ml to 8,000 IU/ml, and most preferably at 6,000 IU/ml. It will be evident that these concentrations may be varied to conform to particular incubation media, to different lots and preparations of rhIL-2, and to other routine variations in clinical and laboratory procedures. For example, if the T-cell activator comprises monoclonal antibodies such as anti-CD3 and/or anti-CD28 used in conjunction with rhIL-2, then a correspondingly lower concentration of rhIL-2 may be required. T-cell activators other than IL-2 may be employed in the present procedures, so long as the donor's PBL are appropriately activated. Such alternative T-cell activators can include, without limitation, interleukin-1 (IL-1), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-12 (IL-12), interleukin-13 (IL-13), interleukin-15 (IL-15), interferon-alpha (IFN $\alpha$ ), interferon-gamma (IFN $\gamma$ ), tumor necrosis factors such as TNF $\alpha$ , anti-CD3 antibodies including antigen-binding fragments thereof (anti-CD3), anti-CD28 antibodies including antigen-binding fragments thereof (anti-CD28), phytohemagglutinin, concanavalin-A and phorbol esters.

The donor's PBL are incubated in the T-cell activator until a sufficient level of activation is achieved. For example, the cells may be incubated in T-cell activator such as rhIL-2 for 2 to 14 days, preferably for 4 or 5 days. The length of incubation can be varied to accommodate routine variations in temperature, media formulations, normal variations in PBL responsiveness, use of additional cytokines required to optimize cell growth and activation, and other routine variables, provided that the PBL attain an appropriate state of activation. For example, if relatively large numbers of

cells are desired for infusion into the patient, then a correspondingly lengthened incubation time may be required. Various laboratory tests may be used to determine an appropriate end-point for the in vitro activation period. These could include fluorescence-activated cell sorting (FACS) to detect various relevant T-cell phenotypes, and measurement of cytotoxic T lymphocyte precursor activity (CTLp).

To diminish or eliminate the possibility of GVHD, allogeneic donor lymphocytes that are life-span-limited may be used. For example, donor lymphocytes may be transduced with a susceptibility factor, or "suicide gene," that makes the lymphocytes susceptible to a chemotherapeutic agent. See, e.g., Tibergheen, *J. Leukocyte Biol.* 56: 203-09 (1994). In one embodiment, thymidine kinase from herpes simplex virus (HS-tk) is employed as the suicide gene. Cells expressing HS-tk are sensitive to killing by exposure to acyclovir or ganciclovir. The HS-tk gene may be transferred into T cells via a retroviral vector containing appropriate promoters, selectable markers and/or other flanking elements. Tibergheen et al., *Blood* 84: 1333-41 (1994); Mavilio et al., *Blood* 83: 1988-97 (1994). Following infusion into the patient, such lymphocytes can be selectively killed, after an anti-tumor effect has been engendered but before onset of severe GVHD. Other methods for limiting the lifespan of the infused allogeneic lymphocytes can include without limitation irradiation, photosensitization and use of anti-lymphocyte antibodies.

The exact number of allogeneic lymphocytes infused may depend on availability and on the patient's previously identified risk factors for GVHD. For example, the patient can be started with  $10^5$  cells/kg, with escalation by one (1) log increment every 1-4 weeks if no GBDH develops following the previous administration. To allow for continued activation of the allogeneic lymphocytes after infusion into the patient, T-cell activator such as IL-2 can be administered to the patient by subcutaneous injection or any other method appropriate for routine drug delivery. This in vivo administration of T-cell activator is preferably initiated on the same day as infusion of the allogeneic lymphocytes, or can be initiated at any time up to about 7 days after infusion. The in vivo administered T-cell activator can be given over a time course of 1 to 14 days, preferably over a time course of 2 to 4 days, and most preferably for 3 days. In a preferred embodiment, rIL-2 is infused into the patient for three days at a concentration of  $10^6$  to  $10^7$ , preferably  $6 \times 10^6$ , IU/ $m^2$  of body surface area. The time course and concentrations can be varied to conform with clinical indications such as propensity for GVHD or ability of the patient to tolerate the chosen T-cell activator.

Following the Allo-CT and/or Allo-ACT treatment and, if desired, in vivo treatment with T-cell activator, the patient is monitored for signs and symptoms of GVHD and, where appropriate, for levels of residual malignant cells. Monitoring for levels of residual malignant cells can involve clinical monitoring of the patient for physical symptoms of relapse. Preferably, the monitoring involves evaluation of diagnostic criteria allowing detection of malignant cells prior to manifestation of physical symptoms. For example, cytogenetic studies may be performed in which macroscopic chromosomal morphology is examined. Alternatively, the monitoring can include the use of molecular probes to detect, for example, aberrant nucleic acid sequences characteristic of the malignant cells. In the case of CML, the patient can be monitored for evidence of the Ph chromosome as revealed by cytogenetic screening or as revealed by PCR analysis of the bcrabl transcript in preparations of nucleic acid taken

from the peripheral circulation. Other disease-specific markers can be equally useful, such as the alpha-RAR marker for AML-M3 as well as a variety of markers developed for solid tumors of varied origin. Disappearance of the selected markers is an indication that the patient has entered remission as a result of the Allo-CT or Allo-ACT treatment regimen.

In the absence of disease-specific markers, other markers can provide equally useful information about the status of host-derived vs. donor-derived cells. For example, the presence or absence of sex-chromosome-specific markers in the host's circulation can be used to monitor female-to-male or male-to-female host/donor combinations. Likewise, the presence or absence of host-specific bands following VNTR (variable nuclear tandem repeat) searching is equally indicative of the effectiveness of cell therapy.

The invention will be further understood with reference to the following illustrative embodiments, which are purely exemplary, and should not be taken as limiting the true scope of the present invention as described in the claims.

#### EXAMPLE 1

##### Induction of a Graft-Versus-Tumor Effect in a Murine Model of Mammary Carcinoma Materials and Methods Mice

BALB/c (H-2<sup>d</sup>) and F1(BALB/cXC57Bl/6)(H-2<sup>d/b</sup>) mice aged 10-12 weeks, DAB/2 (H-2<sup>d</sup>) and C57Bl/6 (H-2<sup>b</sup>) mice aged 7-9 weeks were obtained from Harlan Sprague Dawley, USA, and maintained in a specific-pathogen free animal house at the Hebrew University Hadassah Medical School, according to Israel-specific national laws.

Tumor 4T1 is one of a series of subpopulations isolated from a single, spontaneously arising mammary tumor of a BALB/cC3H mouse. Dexter et al, *Cancer Res.* 38: 3174-81 (1978). It is maintained by passage *in vitro* in RPMI 1640 medium containing 10% heat-inactivated Fetal Bovine Serum (FBS) (Grand Island Biological Co., Grand Island, N.Y.), 2 mM glutamine, 100 mg/ml streptomycin, 100 U/ml penicillin and 1% nonessential amino acids. Preparation of cells for injection includes harvesting by 0.25% trypsin in 0.05% EDTA, washing with RPMI 1640 and resuscitating in Hank's medium for intradermal (ID) injection into mice in a volume of 0.1 ml. All tissue culture media and reagents were purchased from Biological Industry, Beit Ha'emek, Israel. Cells were kept at 37° C. in a humidified 5% CO<sub>2</sub>/air incubator.

##### Measurement of Primary Tumor Growth *in vivo*

Tumor size was measured once a week in two perpendicular dimensions with a caliper. Tumor size in cm<sup>3</sup> was calculated by the formula  $(a \times b)^2/2$  where a is the larger and b is the smaller dimension of the tumor.

##### Flow Cytometric Analysis

A quantity of  $5 \times 10^5$  cells was stained directly with the following monoclonal antibodies: Fluorescein-Isothiocyanate (FITC) anti-H-2K<sup>d</sup> and R-phycocerythrin (PE) anti-LA<sup>d</sup> (Pharmigen, CA, USA). Indirect staining was carried out using rat anti-mouse ICAM-1 (Takei, J. Immunol. 134: 1403-07 (1985)), VCAM-1 (Miyake et al., J. Exp. Med. 173: 599-607 (1991)), CD44 (Trowbridge et al., Immunogenetics 15: 299-312 (1982)) and B7-1 (Razi-Wolf et al., Proc. Natl. Acad. Sci. USA 89: 4210-14 (1992)) antibodies. An FITC-conjugated affinity-purified fragment (Fab)2 of mouse anti-rat IgG, was used as a secondary antibody (Jackson ImmunoResearch Laboratories Inc., PA,

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USA). All staining procedures were carried out on ice for 30 min., followed by washing with phosphate-buffered saline containing 1% bovine serum albumin and 0.03% sodium azide. Cells were fixed in 1% paraformaldehyde and analyzed by FACScan cytometry using the Lysys II program (Becton Dickinson, Santa Clara USA).

### Immunization Protocol

Cultured 4T1 cells ( $10^7$ ) were irradiated (120 Gy) to ensure the absence of proliferating cells from the immunodose, then injected intradermally (ID) into naïve BALB/c mice 3 times in intervals of 7–10 days. Seven to 10 days following the last immunization dose, a challenge of  $10^5$  fresh nonirradiated 4T1 cells was given ID. A control group of naïve nonimmunized BALB/c mice as inoculated in parallel with  $10^5$  fresh 4T1 cells.

### Induction of Bone Marrow Chimeras

Female BALB/c mice were exposed to a lethal dose of 9 Gy total body irradiation (TBI) 24 hr before intravenous injection with  $10^7$  bone marrow cells derived from male DBA/2 mice. Female F1(BALB/cxC57Bl/6) mice were exposed to a lethal dose of 11 Gy TBI 24 hr before intravenous injection with  $10^7$  bone marrow cells derived from male C57Bl/6 mice. TBI was delivered by linear accelerator at an energy of 6 mev. with a dose rate of 1.9 Gy/min. The bone marrow cells were prepared by flushing RPMI 1640 medium through the shafts of the femora and the tibia of the donors with a 25-gauge needle.

### Polymerase Chain Reaction (PCR)

PCR was carried out as previously described. Pugatsch et al., *Leukemic Res.* 17: 900–1002 (1993). Briefly, blood samples were lysed in sterile distilled water and centrifuged for 10 sec at 12,000 g in an Eppendorf centrifuge. Supernatants were discarded and 50 ml 0.05M NaOH were added to the cell pellets. Samples were boiled for 10 min. and 6 ml 1M Tris, pH 7.2, were added. Samples were centrifuged for 5 min. at 12000 g, and care was taken to use only the supernatants for the assay. Oligonucleotide primers were chosen according to the published sequence of a y-chromosome-specific gene (Gubay et al., *Nature* 346: 245–50 (1990)) from position 22–39 for the 5' primer and 342–359 for the 3' primer, respectively. DNA was amplified in a MIR-Mini Cycler in a total volume of 50 ml. Primers were added at a final concentration of 100 pmol and Taq DNA polymerase (Appligene, France) to 1 U/sample. The following program was used: 94° C., 30"; 50° C., 45"; 72° C., 1'; for a total of 35 cycles. Reaction products were visualized on 1.6% agarose gels (Sigma, St. Louis, USA) containing 0.05% ethidium bromide.

### Results

#### Phenotypic Analysis of 4T1 Cell Surface Markers

Phenotypic characterization of 4T1 murine mammary cell surface markers was carried out by using flow cytometry FACS analysis as described above. 4T1 cultured cells express H-2<sup>d</sup> class I antigens (93%) as well as adhesion molecules like ICAM, VCAM (64%, 59%, respectively) and the CD44 homing-associated adhesion molecules (76%). 4T1 cells do not express I-A<sup>d</sup> class II antigens, or costimulatory molecules like B7-1.

#### 4T1 Tumorigenicity

The ability of H-2<sup>d</sup>/4T1 cells of BALB/c origin to form tumors in H-2 compatible as well as incompatible hosts was tested. Intradermal inoculation of  $10^4$  4T1 cells into syngeneic H-2<sup>d</sup> BALB/c mice resulted in a measurable local tumor in 100% of the mice within 21 days. The primary tumor finally led to lung metastases and death of all mice within a median of 39 days (FIG. 1). A delayed appearance of focal tumor was observed only in a fraction of the

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BALB/c hosts (44%) following inoculation of  $10^5$  4T1 cells. Intradermal inoculation of  $10^5$  4T1 cells into congenic H-2<sup>d</sup>/DBA/2 mice caused local tumor and death in only 20% of the mice, while a lower cell dose ( $10^3$ ) led to a transient local tumor that regressed 28 days following tumor inoculation. A measurable primary tumor and lung metastases appeared in 84% of semi-allogenic H-2<sup>d/d</sup> hosts mice within 38 days following inoculation of  $10^4$  4T1 cells. All H-2<sup>d/d</sup> hosts developed tumor died within a median of 50 days (FIG. 1). Inoculation of H-2<sup>d</sup> 4T1 cells into allogeneic H-2<sup>c</sup> C57Bl/6 mice failed to cause tumor in any of the hosts even at a cell dose of  $5 \times 10^5$ . The results show that 4T1 mammary tumor cells bearing H-2<sup>d</sup> antigens can be highly tumorigenic in fully major histocompatible and haploidentical H-2 hosts (BALB/c and (BALB/cxC57Bl/6)F1, respectively), weakly tumorigenic in minor histoincompatible hosts (DBA/2) and are non-tumorigenic in major histoincompatible hosts (C57Bl/6).

### Immunogenicity of 4T1 Cell

Irradiated 4T1 cells ( $10^7$ ) were inoculated 3 times in intervals of 7–10 days into syngeneic BALB/c mice before challenging with a fresh tumorigenic dose of  $10^4$  4T1 cells. These multiple injections of irradiated 4T1 cells did not induce immune protection against a challenge of non-irradiated 4T1 tumor cells (FIG. 2). All mice died with a large local primary tumor as well as lung metastases in a median of 42 days following challenge inoculation. Naive BALB/c mice inoculated with a challenge dose only died in a median of 45 days. Inoculation of either a lower dose of irradiated cells or the same cell dose given only once or twice, failed to induce tumor immunity (data not shown).

### Induction of BM Chimeras across Minor and Major Histocompatible Antigens

Recipient BALB/c (H-2<sup>d</sup>) and (BALB/cxC57Bl/6) F1 (H-2<sup>d/d</sup>) female mice were reconstituted with male-derived minor histoincompatible DBA/2-derived (H-2<sup>b</sup>) and major histoincompatible C57Bl/6-derived (H-2<sup>c</sup>) bone marrow cells, respectively, following a lethal dose of TBI (data not shown). Induction of hematopoietic chimeras was tested using molecular analysis for detection of male y chromosome sequences in peripheral blood cell samples taken 1, 3, 6 and 9 months following BM reconstitution. Results presented in FIG. 3 show evidence for presence of the y-chromosome marker as early as 1 month following bone marrow cell inoculation and continuation of its dominant presence throughout a period of >280 days in DBA/BALB/c chimeras. A stable hematopoietic chimerism was established with light symptoms of chronic GVHD (fur and slight weight loss) across minor histocompatible antigens and with no GVHD overt symptoms across major histocompatible antigens. Respectively, survival time of 9 DBA/BALB/c chimeras was 261 (median) with a range of 147–341 days and >300 days in 14 C57Bl/6-F1 chimeras.

Tumorigenicity of 4T1 Cells in Hematopoietic Chimeras

4T1 tumor cells bearing H-2<sup>c</sup> histocompatible antigens of BALB/c origin were inoculated intradermally into naïve BALB/c (H-2<sup>d</sup>) mice and into BALB/c chimeras carrying minor histoincompatible hematopoietic cells of DBA/2(H-2<sup>b</sup>) origin. Tumor size as a function of days following tumor inoculation is presented in FIG. 4. A measurable local tumor on day 20 was markedly increased with time up to 1.43 cm<sup>3</sup> in naïve BALB/c mice. A significantly smaller tumor ( $p < 0.01$ ) with a limited growth up to 0.27 cm<sup>3</sup> was observed in chimeric DBA/BALB/c mice. Inoculation of 4T1 cells into naïve F1 (H-2<sup>d/d</sup>) mice and F1 chimeras carrying major histoincompatible hematopoietic cells of C57Bl/6 origin, showed a local primary tumor of 0.26 cm<sup>3</sup> which was further

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increased up to 2.40 cm<sup>3</sup> in naïve F1 mice and was significantly smaller ( $<0.05$ ) with limited growth up to 0.31 cm<sup>3</sup> in chimeric C57Bl/6-F1 mice (FIG. 5).

### EXAMPLE 2

#### Graft-Versus-Tumor Effects in a Murine Model of Leukemia

##### 1. Procedures

Inbred, 8-12 week old male and female BALB/c, C57Bl/6 (B6) and (BALB/cxB6) F1 (F1) mice were purchased from the Jackson Memorial Laboratory, Bar Harbor Me., USA. Mice were kept in small isolated cages (5 animals in each cage) and fed sterilized food and acidic water (pH 3.0) during induction of chimerism. Inoculation of leukemia and post-transplant immunotherapy were carried out in a standard non-isolated animal facility.

BALB/c mice were exposed to a single dose of 10 Gy total body irradiation (TBI) from a gamma 150-A<sup>60</sup>Co source (Atomic Energy of Canada) with a focus to skin distance of 75 cm at a dose rate of 58 cGy/min. Twenty-four hours later, the lethally irradiated mice received 5x10<sup>6</sup> T-cell depleted bone marrow cells from B6 donors via the lateral tail vein. Marrow inocula were enriched for stem cells and depleted of immunocompetent T cells by soybean lectin agglutination, according to Reisner et al. Reisner et al., Proc. Natl. Acad. Sci. USA 75:2933 (1978), with minor modifications as reported in Schwarz et al., J. Immunol 138:460 (1987).

rIL-2 was supplied by Dr. C. R. Franks, EuroCetus BV, Amsterdam, The Netherlands, as 1 mg Proleukin (18x10<sup>6</sup> International Units=3x10<sup>6</sup> Cetus Units). rIL-2 was initially diluted with water for injection and subsequently reconstituted with 5% dextrose.

BCL1 cells were maintained in vivo in BALB/c mice by intravenous passages of 10<sup>6</sup>-10<sup>7</sup> peritoneal blood lymphocytes (PBL) obtained from tumor bearing mice. All recipients of BCL1 cells developed splenomegaly and marked lymphocytosis in the blood at the time they were sacrificed to be used as donors for BCL1 cells in experimental mice. Slavin et al., Cancer Res. 41:4162 (1981). PBL counts of all experimental groups were carried out weekly. Onset of leukemia was defined as PBL counts exceeding 20,000/mm<sup>3</sup>. At the peak of disease PBL counts usually reached >100,000/mm<sup>3</sup>. Survival of BCL1 recipients was monitored daily.

Chimerism (i.e., presence of non-self, i.e., donor, hematopoietic cells in a recipient) was determined 4-9 weeks after BMT from the peripheral blood or spleen cells, as previously described. Lapidot et al., Blood 73:2025 (1989). Chimerism was reconfirmed by assaying PBL using an *in vitro* complement-dependent microcytotoxicity assay, with specific alloantiseria (BALB/c anti-B6 and B6 anti-BALB/c) and rabbit-complement, prior to inoculation with BCL1 cells. The percentage of host- or donor-type cells was determined by the trypan blue dye exclusion assay. The specific alloantiseria were prepared by cross-immunizing mice with a full-thickness skin allograft followed by 6 intraperitoneal injections of 30-50x10<sup>6</sup> donor-type spleen cells given 1-2 weeks apart. Mice were bled and sera were stored at -70° C. Chimerism was tested by typing each lymphocyte sample with both antisera: lymphocytes obtained from F1 recipients were lysed 100% by both BALB/c anti-B6 and B6 anti-BALB/c antisera, whereas lymphocytes obtained from B6-BALB/c chimeras were lysed 100% only by BALB/c anti-B6 antiserum; B6 anti-BALB/c antiserum was used to confirm elimination of host cells. The net percentage of chimerism was calculated as

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follows: percentage of cells lysed following treatment with BALB/c anti-B6 antisera (average of duplicate assays) minus cells lysed following treatment with B6 anti-BALB/c antisera minus cells lysed with complement alone.

##### 2. Results

#### Evidence for Chimerism in BALB/c Mice Transplanted with T-cell Depleted B6 Bone Marrow

As described above, BALB/c mice were lethally irradiated and reconstituted with T-cell depleted B6 bone marrow cells. Chimerism was confirmed by assaying PBL shortly after transplantation and again three months later, immediately prior to inoculation with BCL1 cells. All mice were found to be chimeric. Percentages of donor type cells in the blood ranged between 74 and 100%. None of the chimeras showed any clinical evidence of GVHD and the body weight of chimeras was comparable to the body weight of normal controls (data not shown).

##### Resistance of Chimeras to BCL1

Normal BALB/c mice and B6-BALB/c chimeras were injected intravenously with 10<sup>6</sup> BCL1 cells. All normal BALB/c mice developed leukemia, the majority within less than 40 days (median 21 days), and died, whereas all 10 chimeras tested survived with no evidence of disease for >6 months (FIG. 6). A total dose of 10<sup>6</sup> BCL1 cells is sufficient to cause 100% death from leukemia in normal BALB/c recipients (data not shown). Slavin et al. Cancer Res. 41:4162 (1989).

#### Elimination of Clonogenic BCL1 Cells in B6→BALB/c Chimeras with No GVHD

None of the B6-BALB/c chimeras displayed any clinical evidence of GVHD. In order to follow the fate of large numbers of clonogenic BCL1 cells given to the B6→BALB/c chimeras, adoptive transfer experiments were carried out. 10<sup>5</sup> spleen cells (prepared from a pool of 3 chimeras) were transferred to 10 secondary naïve BALB/c mice 7, 14, and 21 days post-inoculation with 10<sup>6</sup> BCL1 cells (FIG. 7). With the exception of a single mouse (1/30), all adoptive recipients of control spleen cells, obtained from normal mice 1, 2, and 3 weeks following inoculation with BCL1 cells developed leukemia within 37 days and died. Seven of 10 secondary recipients of cells obtained from chimeras inoculated 7 days prior to cell transfer developed leukemia within 44 days. In contrast, none of the adoptive recipients of spleen cells obtained from B6→BALB/c chimeras at 14 and 21 days post-inoculation with BCL1 developed leukemia when monitored for more than 6 months. The data suggest that a period of at least 14 days is required for complete eradication and/or inactivation of 10<sup>6</sup> BCL1 cells, whereas at 7 days eradication of leukemic cells is still incomplete.

#### Amplification of GVL Effects by Immunocompetent Allogeneic Spleen Cells and rIL-2 Therapy in Chimeras Inoculated with BCL1 Cells

Twenty-four normal BALB/c mice and 24 well-established B6-BALB/c chimeras were injected with 10<sup>6</sup> BCL1 cells. Injected chimeras were divided into 4 groups. (A) B6→BALB/c chimeras serving as controls with no additional therapy; (B) B6→BALB/c chimeras receiving rIL-2 (10,000 IU x3/day intraperitoneally for 5 days) starting one

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day following inoculation with leukemic cells; (C) B6→BALB/c chimeras receiving 10<sup>7</sup> normal immunocompetent B6 spleen cells; (D) B6→BALB/c chimeras receiving both 10<sup>7</sup> normal B6 spleen cells and rHL-2. For comparison, several controls were included: (E) a control group of normal BALB/c mice inoculated with 10<sup>6</sup> BCL1 cells with no additional therapy; normal BALB/c mice inoculated with 10<sup>6</sup> BCL1 cells received either rHL-2 (F) or allogeneic spleen cells (G) or both (H). Seven days later all mice were sacrificed and their spleen cells were used for adoptive transfer experiments to assess the presence of clonogenic BCL1 cells. Secondary BALB/c recipients (5 in each group) received 10<sup>5</sup> spleen cells obtained from a pool of 3 control BALB/c mice or from 3 B6→BALB/c chimeras of each experimental group. Matching results were obtained when the experiment was duplicated with the remaining three mice of each group. The data were therefore pooled and each experimental group shown in FIG. 8 consists of 10 mice.

All secondary BALB/c recipients receiving spleen cells obtained from normal BALB/c mice (E) developed leukemia within 32–37 days. Seventy percent of secondary recipients receiving spleen cells obtained from B6→BALB/c chimeras (group A) did not develop leukemia for >6 months, whereas in the 30% that did develop leukemia, the onset of disease was delayed (onset within 44–52 days). B6→BALB/c chimeras treated with rHL-2 (B), allogeneic immunocompetent donor-type splenocytes (C) or the combination treatment of both (D) displayed marked resistance against leukemia, with no evidence of spleen disease for >9 months in all secondary recipients of spleen cells obtained from groups B and D and with delayed onset of leukemia in only 20% of mice receiving spleen cells from group C. No anti-leukemia effects were detected in normal control BALB/c mice treated with rHL-2, allogeneic splenocytes or both (F, G, and H, respectively).

#### EXAMPLE 3

##### Clinical Results

###### 1. Breast Cancer

A 40 year old female patient presented who had developed an upper medial quadrant mass in the left breast at the age of 37. Physical examination revealed an undefined mass at that region and a 4×3 cm mass in the left axilla. Excisional biopsy was taken from the breast mass and the pathological examination revealed a grade III multifocal infiltrating ductal carcinoma with three masses of 3×2×2, 1.5×1×1 and 1×1×1 cm in size. In addition, there was tumor invasion to the lymphatic vessels with a positive surgical margin. The patient was treated with 7 cycles of CAF (Cyclophosphamide, Adriamycin and 5 Fluor-uracil). Chemotherapy was followed by left upper quadrantectomy and axillary lymph node dissection. The pathological report from this specimen revealed three residual foci of infiltrating ductal carcinoma of 1×1×1, 1×1×1.5 and 0.5×0.5×0.5 cm in size. Three out of 17 nodes were involved with cancer. The patient completed 56 Gy breast irradiation followed by a 14 Gy boost dose to the tumor area using 12 MeV electron beam irradiation.

Twenty three months later a 1.5 cm mass was noted in the medial aspect of the quadrantectomy scar adherent to the chest wall. FNA aspiration was performed and cytological analysis revealed malignant cells which were consistent with breast cancer. Blood count at that time showed Hgb of 7.5 g % and WBC of 1.3×10<sup>9</sup>/L. Bone marrow biopsy was performed and the diagnosis was compatible with AML-M2. Analysis of the phenotype of the blast cells by fluorescence

activated cell sorter showed HLA-DR 76%, CD34 65%, CD33 66%, CD13 56%, CD15 41%, CD11B 40% and CD11C 80%. Systemic evaluation included whole body CT scan, abdominal ultrasound, liver scan, bone scan, CA-15-3 and CEA; all were within the normal range. The patient was treated with one cycle of amscarine and high dose cytosar with subsequent disappearance of blast cells in bone marrow. A slight decrease in the size of the chest wall mass was noted.

Four months following the diagnosis of AML, the patient underwent a T cell depleted allogeneic stem cell transplantation from a full HLA A, B, C, DR and DRB1-matched MLR non-responsive brother. The conditioning protocol included pretransplant immunosuppression with anti-thymocyte globulin (Preseris) 10 mg/kg for 4 consecutive days and subsequent administration of busulfan 4 mg/kg/day×4, thiotope 10 mg/kg/day×1, cytoxan 50 mg/kg/day×4 and intrathecal ARA-C for CNS disease prophylaxis. T cell depletion was accomplished by adding monoclonal rat anti-human lymphocyte (CDw52) antibody (Campath-1G, provided by Dr. G. Hale, Oxford University, UK) at 0.3 ug/10<sup>6</sup> nucleated cells to the bag containing the marrow cells as previously described (Naparstek et al., *Exp. Hematol.* 17: 723 (abstr.) (1989)). Engraftment (ANC>0.5×10<sup>9</sup>/L; PLT>2×10<sup>9</sup>/L) was documented on the 21st day following transplantation. Following transplantation the cytogenetic studies revealed full reconstitution with donor-derived cells in the blood.

Ten weeks following the transplantation there were no clinical signs of GVHD; hence, the patient was treated with allogeneic cell-mediated immunotherapy (allo-CT) consisting of donor blood lymphocyte infusion at a cell dose equivalent to 1×10<sup>5</sup> T cells/kg. Four weeks later a transient impairment of liver function tests (GGTP 712, ALT 301, and AST 258 Units) was observed. No other clinical findings indicative of GVHD were noted. Twenty weeks post transplant, a higher dose of donor blood lymphocytes consisting of 0.6×10<sup>7</sup> T cells/kg was given. At greater than 8 months post-transplant, the patient is event free with no evidence of either breast cancer or AML. No evidence of acute or chronic graft vs host disease (GVHD) developed although the patient received no anti-GVHD prophylaxis.

The present inventor is not aware of any cases in which a patient with such an aggressive recurrent breast cancer

went into stable complete response after receiving only "suboptimal" chemotherapy as was used in this patient.

###### II. Hematologic Malignancies

Patient No. A 17 year-old man with CML in accelerated phase was admitted to Hadassah University Hospital Department of Bone Marrow Transplantation for allogeneic BMT. The patient had an HLA-A, B, DR, DRB1 matched brother non-reactive in bilateral mixed lymphocyte culture for allogeneic BMT. Pre-transplant cytogenetic analysis of the patient disclosed 100% Ph<sup>+</sup> positivity in bone marrow spontaneous metaphases with three different malignant spontaneous clones: 35% 46XX (q:22); 35% 46XY (q:22) add (15) (q26); 30% 46XY (q:22 add (2) (q37). Additionally, the patient was classified as 100% positive for the bcr/abl fusion product, as detected by PCR in a peripheral blood sample. Pre-transplant conditioning included cyclophosphamide (60 mg/Kg×2 days) and total body irradiation (200 cGy daily×6 days).

On Jul. 21, 1993, he was transplanted with 2.5×10<sup>6</sup> viable nucleated cells/kg (non-T-cell depleted) from his compatible brother. He was treated with cyclosporin A (starting day -1) and methotrexate (days 1, 3, 6 and 11) as anti-GVHD prophylaxis as previously described. Goldman, Leuk. and

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Lymph. 3: 159-64 (1990). The graft/graft was normal with white blood cell (WBC) count  $>1 \times 10^9/L$  on day +26, neutrophil count  $>5.5 \times 10^9/L$  on day +25 and platelet count  $>25 \times 10^9/L$  on day +25. He was discharged 24 days post BMT. At one month post BMT, PCR disclosed no bcr/abl fusion product. Cyclosporin A was tapered off and discontinued 3 months post BMT. A month later, at 4 months post BMT, the PCR converted to bcr/abl positivity and marrow cytogenetic analysis revealed 100% Ph+ with clonal selection. Marrow morphology was compatible with chronic phase CML. No significant increase in peripheral blood counts was noticed.

In an attempt to reinduce remission he was treated with allogeneic cell-mediated immunotherapy (Allo-CT) using the compatible brother's peripheral blood lymphocytes (PBL) ( $8.9 \times 10^9$  cells infused/kg). Two weeks later, in the absence of any sign of GVHD, the patient was given another infusion of PBL ( $5 \times 10^9$  cells/kg) with *in vivo* rhIL-2 ( $3 \times 10^6$  IU/m $^2$ ) given subcutaneously for 3 consecutive days on an outpatient basis. No signs of GVHD developed. There was a transient decline in the WBC counts from  $14.7 \times 10^9/L$  to  $6.2 \times 10^9/L$  with no change in the hemoglobin and platelet counts. The PCR for the hcr/abl fusion product remained positive. With continued evidence of malignant cells following BMT and Allo-CT, the patient's prognosis was very poor absent additional therapeutic measures.

In an attempt to escalate the therapeutic regimen, PBL from the compatible brother were precultured in RPMI medium (Belt Hseme-K, Israel) supplemented with 5% inactivated autologous AB serum and further supplemented with 6,000 IU/ml of rhIL-2. The PBL were maintained in the medium for 4 days in a humidified 5% CO<sub>2</sub> in air incubator, at a concentration of  $2.5 \times 10^6$  cells/ml. After 4 days incubation of an initial cell dose of  $17 \times 10^9$  viable cells, a total of  $28 \times 10^9$  ADL were harvested.

In January 1994, the patient received  $3.7 \times 10^7$  allogeneic ADL/kg, together with 3 days administration of subcutaneous rhIL-2 ( $3 \times 10^6$  IU/m $^2$ ), beginning on the same day as the ADL administration, for further *in vivo* activation of the allogeneic ADL. The WBC dropped from  $11.3 \times 10^9/L$  to  $1.3 \times 10^9/L$ . Hemoglobin dropped from 11.5 g % to 8.3 g % and the platelet count dropped from  $346 \times 10^9/L$  to  $23 \times 10^9/L$ . PCR became negative for the bcr/abl fusion product. Bone marrow cytogenetic analysis detected 100% normal male karyotype in all spontaneous metaphases. Bone marrow morphology was completely normal and there were no clinical laboratory signs of GVHD. Blood counts improved gradually with no further therapy. At 2 months following Allo-ACT, at the time the WBC count was  $2.8 \times 10^9/L$ , platelet  $78 \times 10^9/L$  and hemoglobin 10.2 g %, the patient developed disseminated herpes zoster with abnormal liver function tests including bilirubin 17 (normal range 2.5-17) micromol/L, AST 444 (normal range 7-40) units, ALT 561 (normal range 6-53) units, GTP 346 (normal range 60-170) units. The patient is more than 28 months post Allo-ACT with no evidence of the Ph+ clone (by both cytogenetics and PCR analysis) and with no signs of severe GVHD and good general condition.

Patient No. 2. A five-year-old boy was diagnosed with called primary acute lymphocytic leukemia (ALL) in 1988. Allogeneic BMT was performed in Barcelona, Spain on Jan. 8, 1990. At the time of BMT, the patient was in a second complete remission. The conditioning regimen consisted of cyclophosphamide, 60 mg/kg on two consecutive days, plus fractionated total body irradiation (TBI), 200 cGy $\times$ 6 (a total of 1200 cGy). The donor bone marrow was from a fully

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matched brother, and was given without T-cell depletion. The patient was given standard post-transplant anti-GVHD prophylaxis with cyclosporin A. Following BMT, Grade 1 GVHD developed. Overt hematologic and cytogenetic relapse was diagnosed one month later with T(2;3), (Q3;P14), DEL(13) (Q?), DEL (20) (Q11) clones.

The patient received post-transplant Allo-CT consisting of the donor's PBL at an equivalent dose of  $1.4 \times 10^9$  T cells/kg given on Oct. 8, 1991, followed on Nov. 3, 1991 by  $3.5 \times 10^9$  T cells/kg with concomitant administration of rhIL-2 subcutaneously ( $6 \times 10^6$  IU/m $^2$ ) for 3 consecutive days starting on the day of cell infusion. Subsequently, in December of 1991, the patient received  $3 \times 10^9$  ADL/kg prepared by treating donor PBL *in vitro* with 6,000 IU/ml of rhIL-2 for 4 days. Five days later, the patient developed cutaneous GVHD grade III which responded over the course of one month to corticosteroid therapy. Following the Allo-ACT-*in vivo* T-cell activator regimen, the patient entered a complete remission, documented by a normal cytogenetic pattern observed in all metaphases investigated. Chronic GVHD of the skin has persisted, and the patient remains in complete remission over 53 months post Allo-ACT.

Patient No. 3. A nine-year-old girl was initially diagnosed in September 1990 with adult-type CML, 100% Philadelphia chromosome-positive cells. She underwent allogeneic BMT on Feb. 21, 1991, in Seattle, while in Chronic Phase. Conditioning consisted of cyclophosphamide, 60 mg/kg, on two consecutive days, followed by fractionated TBI, 200 cGy $\times$ 6 (total dose of 1200 cGy). A full HLA AB DR-matched MLR non-reactive brother was the donor, and the donor's cells were not T-cell depleted. The patient received standard anti-GVHD prophylaxis with cyclosporin A, and developed no GVHD. Nine months following BMT, the patient had overt hematologic and cytogenetic relapse with 100% of the observed metaphases revealing the Philadelphia chromosome.

The patient received PBL from the same donor at an equivalent dose of  $5 \times 10^9$  T cells/kg given on Dec. 3, 1991. The donor was less than three years old and, therefore, full phresis was not technically feasible. The patient received a total dose of donor PBL equivalent to  $10^9$  T cells/kg on Jan. 15, 1992, with concomitant administration of rhIL-2 subcutaneously ( $6 \times 10^6$  IU/m $^2$ ) for 3 consecutive days starting on the day of cell infusion. Subsequently, in February of 1992, the patient received ADL at an equivalent dose of  $10^9$  cells/kg. In March of 1992, a second dose of  $10^9$  ADL/kg was administered with concomitant administration of rhIL-2 subcutaneously ( $6 \times 10^6$  IU/m $^2$ ) for 3 consecutive days starting on the day of cell infusion. The ADL were prepared by treating donor PBL *in vitro* with 6,000 IU/ml of rhIL-2 for 4 days.

The patient responded hematologically; however, a reverse transcriptase PCR assay (RT-PCR) indicated the presence of residual Philadelphia chromosome-positive cells. Following treatment with alpha interferon (Roferon A), all cytogenetic abnormalities disappeared as evidenced by a negative RT-PCR assay for the bcr/abl fusion product. The patient showed no evidence of GVHD throughout the treatment. The patient is doing very well over 51 months post Allo-ACT; she is hematologically normal with no abnormal karyotypes and is consistently negative by RT-PCR for the bcr/abl fusion product. She is in excellent clinical condition with no signs of chronic GVHD.

Patient No. 4. A three year old girl was diagnosed with adult-type, Philadelphia chromosome-positive CML in November of 1990. The patient was conditioned for allogeneic BMT with busulfan, 16 mg/kg over four consecutive

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days and with cytoxan, 200 mg/kg over four consecutive days. Cells for allogeneic BMT were taken from a fully matched minor brother (one year old). The BMT was performed, without T cell-depletion, on May 2, 1991, with standard anti-GVHD prophylaxis using cyclosporin A. The patient had an uneventful outcome following BMT, with no GVHD. At 8 months post-BMT, the patient developed overt hematologic and cytogenetic relapse.

Cell therapy consisted of donor PBL from the BMT donor at an equivalent dose of  $5 \times 10^8$  T cells/kg administered in 10 February 1992. A similar dose of donor PBL was administered in March of 1992, with concomitant administration of rhIL-2 subcutaneously ( $6 \times 10^6$  IU/m<sup>2</sup>) for 3 consecutive days starting on the day of cell infusion. In April of 1992, the patient received ADL from the BMT donor at an equivalent dose of  $5 \times 10^8$  T cells/kg. In July of 1992, the patient received ADL from the BMT donor at an equivalent dose of  $3 \times 10^8$  T cells/kg with concomitant administration of rhIL-2 subcutaneously ( $6 \times 10^6$  IU/m<sup>2</sup>) for 3 consecutive days starting on the day of cell infusion.

No signs of GVHD developed and, perhaps consequently, the patient showed progressive disease despite the cellular immunotherapy. Further treatment with Roferon A failed to induce cytogenetic remission. The patient underwent a second allogeneic BMT with no T cell-depletion in September 25 of 1994, but died due to progressive disease.

Patient No. 5. A two year old girl was diagnosed in August of 1992 with myelodysplastic syndrome (MDS) refractory anemia, with excess blasts displaying a clonal (9;11) translocation, evidence of transition to leukemia. Allogeneic BMT from a fully HLA A B DR DRB1-matched and MLR non-responsive brother was carried out on Feb. 19, 1993. Conditioning consisted of busulfan, 16 mg/kg given over four consecutive days, thiogata, 10 mg/kg given over two consecutive days, and cytoxan, 16 mg/kg given over two consecutive days. The allogeneic BMT was non-T-cell depleted, and the patient had a non-eventful outcome with no signs of GVHD following standard anti-GVHD prophylaxis with cyclosporin A. The patient went into full relapse with the same clonogenic leukemia at five months following BMT.

In August of 1993, the patient was treated with donor PBL from the BMT donor at an equivalent dose of  $2.8 \times 10^8$  T cells/kg. No evidence of GVHD developed. In September of 1993, the patient received the same donor's PBL at an equivalent dose of  $4 \times 10^8$  T cells/kg, with concomitant administration of rhIL-2 subcutaneously ( $6 \times 10^6$  IU/m<sup>2</sup>) for 3 consecutive days starting on the day of cell infusion. In November of 1993, the patient received ADL from the BMT donor at an equivalent dose of  $1.4 \times 10^8$  T cells/kg with concomitant administration of rhIL-2 subcutaneously ( $6 \times 10^6$  IU/m<sup>2</sup>) for 3 consecutive days starting on the day of cell infusion. No evidence of GVHD developed. Despite the absence of GVHD, the patient showed a complete hematologic and cytogenetic response with 20 out of 20 metaphase featuring normal male karyotype with no chromosomal aberrations and normal bone marrow morphology. Unfortunately, overt relapse was noted again in January of 1994, and the patient died in February 1994 due to progressive disease.

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What is claimed is:

1. A method of treating a human cancer patient having a solid tumor comprising malignant cells, said patient having undergone a cancer therapy regimen comprising allogeneic stem cell transplantation, said method comprising:
  - a) administering allogeneic lymphocytes to said patient; and
  - b) monitoring said patient for levels of said malignant cells.
2. The method of claim 1, wherein said solid tumor is a breast carcinoma.
3. The method of claim 1, wherein said allogeneic lymphocytes are activated by exposure to a T-cell activator in vitro prior to administration to said patient.
4. The method of claim 3, wherein said T-cell activator comprises at least one T-cell signal transduction pathway activator.
5. The method of claim 4, wherein said T-cell activator is selected from the group consisting of IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, IL-13, IL-15, IFN $\alpha$ , IFN $\gamma$ , TNF $\alpha$ , anti-CD3, anti-CD28, phytohemagglutinin, concanavalin-A and phorbol esters.
6. The method of claim 5, wherein said T-cell activator comprises IL-2.
7. The method of claim 1, wherein said allogeneic lymphocytes are administered to said patient in a series of incrementally increasing amounts, pending no or controllable graft-versus-host disease between increments.
8. The method of claim 1, wherein said allogeneic lymphocytes are HLA-compatible with said patient.
9. The method of claim 1, wherein said administration of allogeneic lymphocytes is accompanied by in vivo administration of T-cell activator.
10. The method of claim 9, wherein said in vivo administered T-cell activator is given to said patient over a time course of two to four days.
11. The method of claim 9, wherein said T-cell activator comprises at least one T-cell signal transduction pathway activator.
12. The method of claim 11, wherein said T-cell activator is selected from the group consisting of IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, IL-13, IL-15, IFN $\alpha$ , IFN $\gamma$ , TNF $\alpha$ , anti-CD3, anti-CD28, phytohemagglutinin, concanavalin-A and phorbol esters.
13. The method of claim 12, wherein said T-cell activator comprises IL-2.
14. The method of claim 1, wherein said allogeneic lymphocytes are lifespan-limited.
15. The method of claim 14, wherein said allogeneic lymphocytes carry a suicide gene conferring on said lymphocytes susceptibility to killing by a chemotherapeutic agent following administration of said lymphocytes to said patient.
16. The method of claim 1, wherein said allogeneic lymphocytes are administered to said patient in the form of a peripheral blood mononuclear cell preparation.

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